# Sex as a Biological Variable:
# A Workshop on NIH Grantee Findings

National Institutes of Health
Bethesda, MD

**October 26–27, 2017**

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# EXECUTIVE SUMMARY

## *Background*

For many years, investigators believed that aside from reproductive differences and sex hormones, men and women shared the same biology. Therefore, it was believed that results from the study of one sex could be applied to the other. We now know that some diseases show a sex bias and are unique to or more prevalent in women, while other conditions are more common in men. In addition, each sex can experience different symptoms and be affected by medications in different ways, even with diseases and conditions that are similarly prevalent in men and women.

In 2013, the National Institutes of Health (NIH) Office of Research on Women's Health (ORWH) began a trans-NIH initiative to provide administrative supplemental funding to ongoing peer-reviewed NIH grants, with the goal of increasing the inclusion of both males and females in preclinical and clinical research. In 2014, the NIH Common Fund, which supports the most innovative and high-potential biomedical research at NIH, also adopted the administrative supplement paradigm. Since then, millions of dollars in supplemental funding have been provided to more than 300 grantees across the NIH Institutes, Centers, and Offices (ICOs) to explore research on sex/gender differences in preclinical and clinical studies.

In 2014, the ORWH Director, Janine A. Clayton, M.D., and the NIH Director, Francis Collins, M.D., Ph.D., called on NIH to address an imbalance in preclinical studies caused by findings that were often based on animals of only one sex, typically males.[1] They noted that an overreliance on male animals could obscure the understanding of key sex influences on health processes and outcomes.

Building on these efforts, NIH released the Sex as a Biological Variable (SABV) policy (NOT-OD-15-102)[2] in June 2015, with an effective date of January 2016. Since that time, NIH has expected SABV to be factored into the designs, analyses, and reporting of research on vertebrate animal and human studies. Because consideration of sex can be critical to the interpretation, validation, and applicability of research findings, strong justification from the scientific literature, preliminary data, or other relevant considerations must be provided in grant applications that propose to study only one sex.

On October 26–27, 2017, a workshop was convened with NIH grantees from a variety of disciplines who shared the approaches they used for considering SABV in 16 investigations. They were given the opportunity to present their findings, share lessons learned, and help chart the way forward for other researchers as they incorporate SABV into their work. A panel session followed that provided useful recommendations for encouraging researchers to include both sexes in their studies, to disaggregate data by sex and gender, and to analyze the resulting data in more depth than before.

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[1] Clayton, J.A., Collins, F.S. (2014). Policy: NIH to balance sex in cell and animal studies. Nature, 509(7500):282–283. doi:10.1038/509282a. PMID: 24834516.

[2] https://grants.nih.gov/grants/guide/notice-files/not-od-15-102.html

## *Workshop Topics*

Workshop presentations were grouped into the following four sessions:

- Sex Differences in Brain Function and Behavior
- How Sex Impacts Our Interaction with External Influences
- Sex Differences in Animal Models
- Sex Differences in Gene Expression

## *Session Highlights*

Presenters shared their findings on the influence of sex in a wide variety of scientific areas. Key findings are listed below; they are described in detail in the full report.

- Female rats require twice as much morphine as males to produce comparable levels of analgesia. This may be caused in part by a higher number of activated microglia in certain female brain regions.
- Neurodevelopmental processes can vary by sex. For example, neurulation, myelination, synaptogenesis, and other processes differ between males and females.
- In patients with chronic pain, significant sex differences exist in brain anatomy, connectivity, and responses to pain stimuli.
- In females with schizophrenia, use of atypical antipsychotics is associated with an increased rate of metabolic syndrome.
- In Alzheimer's disease, epigenetic changes in human fibroblasts seem to indicate a different disease mechanism in females when compared with males.
- Postmenopausal female mice were found to catabolize brain myelin, leading to white tissue degeneration, a phenomenon also seen in humans with Alzheimer's disease.
- Similar biological mechanisms may be at work in creating both anthropometric and common disease sex differences in autism.
- Clear differences in obesity rates were shown between African American men and women, as well as between Mexican American men and women. In both groups, more women than men were obese.
- Sex may be an important effect modifier in the resulting titers for some vaccines.
- Gut microbes in male mice can influence protection from type 1 diabetes.
- Testosterone levels in male mice are partially mediated by their gut microbes.
- Estrogen may play an important role in the allergic asthma response in mice.
- Androgen receptor activation can potentiate urinary tract infection (UTI) susceptibility and severity.
- While both hormone levels and sex chromosome genes are involved in sex differences, autosomal genes also play a role in sex differences.

## *Conclusions*

Workshop participants highlighted many important ways that sex acts as a *fundamental* biological variable across body systems and disease categories. They presented a broad spectrum of creative and effective approaches for considering SABV, depending on research context and model system, thus

making clear that there is no one-size-fits-all approach to integrating SABV into biomedical research. Despite the diversity of approaches, several key themes emerged from the workshop participants sharing their lessons learned, resources, and data sets, all of which were helpful to other investigators who are considering how to include SABV in their research efforts.

It was clear from the presentations and discussions that researchers benefit a great deal from considering SABV in the early phases of planning and designing their research. Results of epidemiologic studies can inform how basic and preclinical investigations are designed to consider SABV, and vice versa. Not all studies need to be designed and powered to test for sex influences for them to consider SABV. Researchers must include both sexes in their research unless there is strong scientific justification not to do so, and whenever both sexes are included in the research, data should be presented separately for females and males, at a minimum. Workshop participants underscored the importance of making raw data available, when feasible, or reporting data disaggregated by sex. Results presented this way may be used in subsequent meta-analyses and can also inform the design of follow-up research that includes planned and appropriately powered tests for sex influences.

Several presenters confirmed that when their data were aggregated across sexes, no sex influences were apparent. Stratifying their data by sex or performing exploratory statistical tests of sex influences often suggested potentially important sex effects, which would have otherwise remained hidden in the aggregate data. One of the workshop participants also showed how similar outcomes between the sexes can arise from different pathogenic processes in males and females. Such mechanistic differences underlying trait similarities can have important implications for the development of sex-aware treatments.

During the workshop, participants emphasized the important roles that different stakeholders may play in communicating sex-based results and strengthening the translation of those results into improved health care for women and men. Researchers have a central role in this, but journal editors and the broader medical community, including professional societies, have essentials roles to play as well. Scientists whose research has benefited from the consideration of SABV may serve as SABV ambassadors and positively influence their colleagues' attitudes about SABV, just as patients may increase the demand for sex- and gender-aware treatment approaches for their own health care. Some workshop participants also emphasized the value of educational resources for expanding the understanding of SABV among reviewers of grant applications, as well as graduate students.

The presentations given at the 2017 SABV Workshop showed how preclinical models are beginning to provide mechanistic insights into sex differences that are needed to guide the development of optimal human therapies for women and men. Sixteen compelling examples of the benefits of incorporating SABV into research were presented. Considering SABV in experimental design and analyses is not only an NIH policy. It is also a bedrock of valid science, as was well illustrated by the workshop participants.

# INTRODUCTION

***Janine A. Clayton, M.D., Director, NIH Office of Research on Women's Health (ORWH)***
***Elizabeth Wilder, Ph.D., Director, NIH Office of Strategic Coordination (OSC) – The Common Fund***

Dr. Elizabeth Wilder, whose office manages the NIH Common Fund, explained that the Common Fund is a component of the NIH budget that supports approximately 30 programs intended to transform health, knowledge, and the way in which biomedical research is conducted. In pursuit of this transformation, NIH often develops new tools, technologies, and large data sets that would be difficult to generate without a dedicated funding source. The Common Fund has supported SABV administrative supplements for its grantees since 2013.

Dr. Janine Clayton explained that one aspect of the mission of the NIH Office of Research on Women's Health (ORWH) is to enhance and expand women's health research. This includes examining sex and gender influences on disease to improve the health of women. She noted that historically, women were excluded from many clinical trials, yet the data from these studies were often applied to women.

In 2013, ORWH initiated a program to incorporate research on sex and gender influences on health and disease into ongoing NIH-funded research by providing administrative supplements. Since the program's inception, ORWH has invested over $30 million and supported more than 300 investigators from most of the NIH Institutes, Centers, and Offices (ICOs). These supplements are complementary to the work of the parent grant. The supplements add to the study questions posed in the parent grant proposal rather than change their direction. The goal of the administrative supplement program has been to build a robust body of evidence on male and female biology that will inform prospective research.

This workshop was convened to highlight studies from researchers at the forefronts of their fields who received supplemental funding from ORWH or the Common Fund. The four research areas represented are (1) sex influences on brain function and behavior, (2) how sex impacts our interaction with external influences, (3) sex differences in animal models, and (4) sex differences in gene expression.

# KEYNOTE ADDRESS: SEX-SPECIFIC RISK FOR CARDIOVASCULAR DYSFUNCTION AND COGNITIVE DECLINE

***Virginia M. Miller, Ph.D., Mayo Clinic, Rochester, MN***

Dr. Virginia Miller presented studies conducted through the Specialized Centers of Research (SCOR) on Sex Differences program related to pregnancy complications, oophorectomy (surgically induced menopause), natural menopause, and hormone replacement. Dr. Miller explained that hormones can sometimes play a role in sex differences.

The first study focused on pregnancy history and the risk of future cardiovascular disease. Researchers recruited women from the Rochester Epidemiology Project, a collaboration of clinics, hospitals, and other medical facilities in Minnesota and Wisconsin. The project involves community members who have agreed to share their medical records for research purposes.

Women with a history of hypertensive pregnancy (preeclampsia) were compared with women who had a history of normotensive pregnancy. Imaging was used to detect coronary artery calcification, carotid

intima-media thickness, cerebral blood flow, and brain atrophy. Results indicated that women with a history of preeclampsia showed significantly more coronary arterial calcification and greater carotid intima-media thickness than women with a history of normotensive pregnancy. (See Figure 1.) In addition, women with a history of preeclampsia had decreased cerebrovascular reactivity to a carbon dioxide provocation and showed significant atrophy in occipital brain areas and impaired visual/spatial function. The findings suggest a potential benefit for increased monitoring after a hypertensive pregnancy. In addition, the investigators suggested that patient questionnaires include a section for pregnancy history and complications to help doctors assess the risk for chronic disease and determine the need for long-term care.

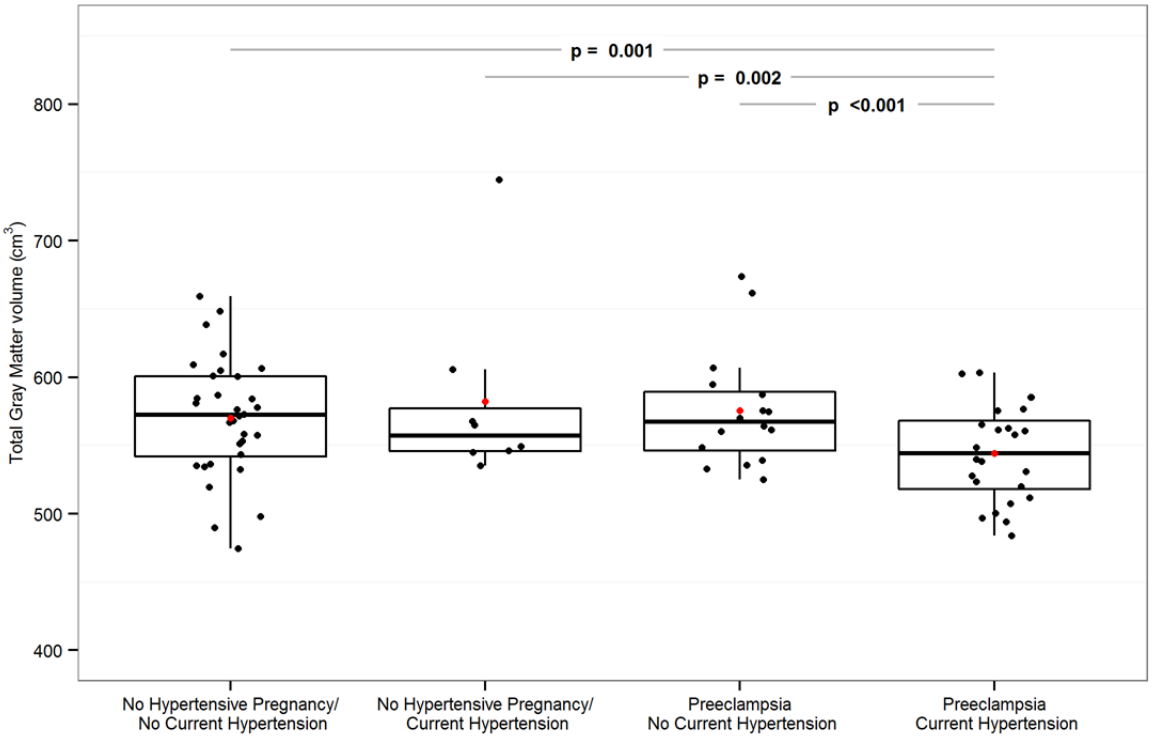


**Figure 1.** Group differences in gray matter volume in women with histories of normotensive or preeclamptic pregnancy with and without current hypertension. Individual data points are shown with median and 25th and 75th percentile. Red dots indicate mean for each group. Derived from Figure 1 of Raman, M. R., Tosakulwong, N., Zuk, S. M., Senjem, M. L., White, W. M., Fields, J. A., et al. (2017). Influence of preeclampsia and late-life hypertension on MRI measures of cortical atrophy. *J Hypertens*, *35*, 2479–2485.

A second study of oophorectomies also evaluated data from the Rochester Epidemiology Project. A total of 1,031 women who had bilateral oophorectomies at 45 years of age or younger were compared with a control group who did not have oophorectomies. The findings indicated that women who had bilateral oophorectomies experienced accelerated aging compared with the control group, as shown by a greater incidence of 18 chronic conditions. None of the women studied had these conditions prior to their surgeries. Within 6 years of having an oophorectomy, there was an accumulated acceleration of the number of comorbidities compared with the reference women. (See Figure 2.) For example, women who had oophorectomies had increased cardiovascular mortality. However, when women who had oophorectomies were treated with estrogen, they showed improved survival rates compared with women who received no treatment. Clinical practice now requires that women who have an

oophorectomy before the age of 45 be treated with estrogen. Dr. Miller noted that an oophorectomy should not be considered for benign conditions.

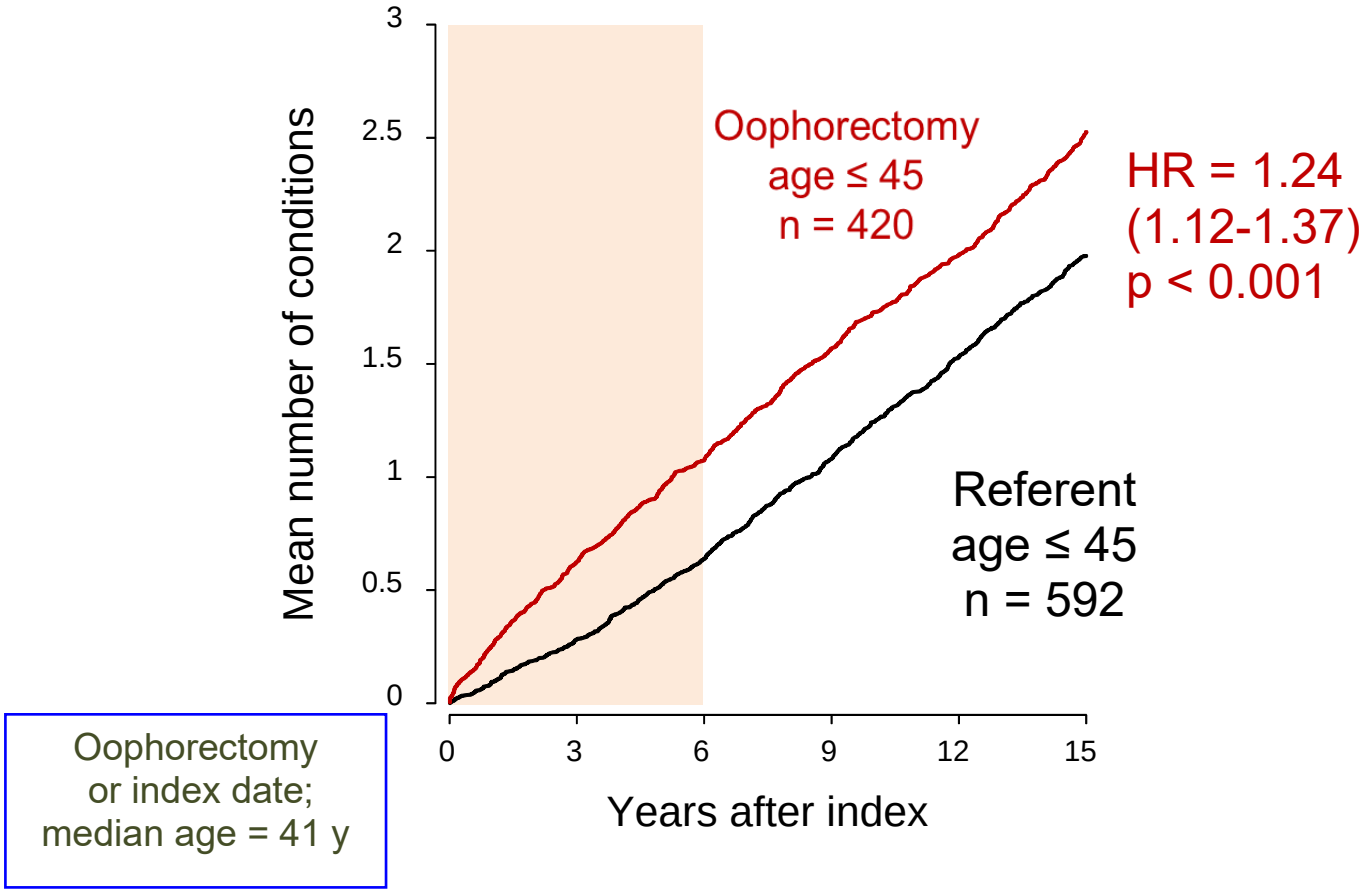


**Figure 2**. Mean number of conditions (multimorbidity) in women who underwent bilateral oophorectomy prior to the age of 45 years compared with age-matched group of women who did not undergo the procedure (referent). The accumulation of conditions in the shaded area was significantly greater within 6 years of the procedure than for the referent women and paralleled thereafter. The hazard ratio (HR) was adjusted for education, body mass index, smoking, and age. Derived from Figure 1 of Rocca, W. A., Gazzuola-Rocca, L., Smith, C. Y., Grossardt, B. R., Faubion, S. S., Shuster, L. T., et al. (2016). Accelerated accumulation of multimorbidity after bilateral oophorectomy: a population-based cohort study. *Mayo Clin Proc*, *91*, 1577–1589.

A third study examined women who experienced natural menopause, using data from the Kronos Early Estrogen Prevention Study (KEEPS). Women who did not have preexisting cardiovascular disease and who experienced menopause between the ages of 42 and 58 were randomized to a placebo, conjugated equine estrogen, or an estradiol patch. They were given natural oral progesterone for the first 12 days of the month for 4 years. Both treatment groups showed positive changes in bone mineral density and reduced symptoms associated with menopause (e.g., hot flashes, night sweats, sleep disturbances) and had improved sexual function. Changes in carotid intima-media thickness were similar among the groups during the study. The investigators believed this was because the women had a low cardiovascular risk and the study lasted only 4 years.

Subsequent research involving a group of participants in KEEPS showed significantly lower brain amyloid deposition in women with the ApoE4 allele who used the estradiol patch than in those who took a

placebo. (See Figure 3.) The ApoE4 allele is a genetic risk factor for Alzheimer's disease. In order to be confirmed, this observation would require testing in a larger study.

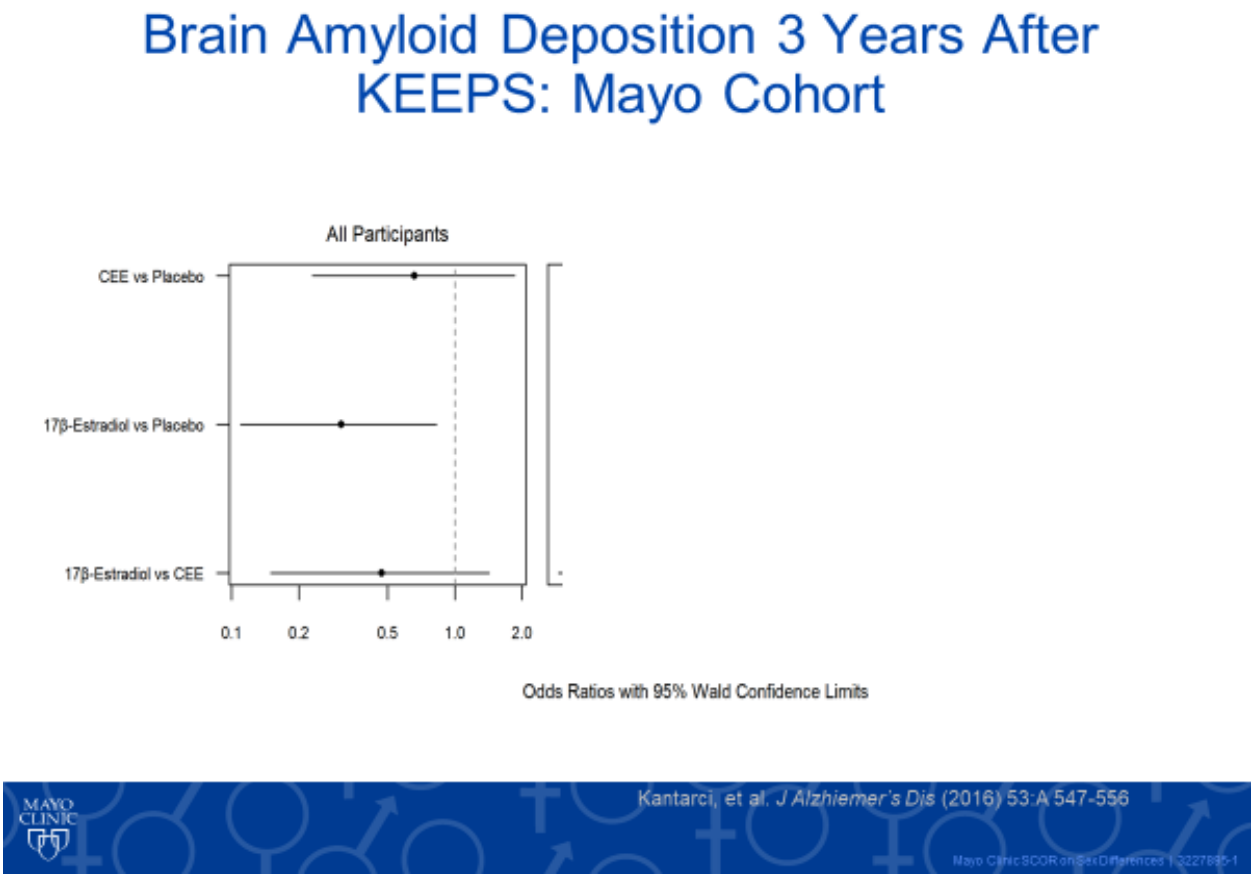


**Figure 3**. Odds ratios for accumulation of amyloid-β in the brains of menopausal women who participated in the Kronos Early Estrogen Prevention Study (KEEPS) and who were randomized to conjugated equine estrogen (CEE), transdermal 17β-estradiol, or a placebo for 4 years. Imaging was performed 3 years after cessation of treatment. The odds ratio was derived using proportional odds logistic regression models and 95% Wald confidence limits in all participants, ApoE ε4 carriers and non-carriers after adjusting for age. The odds ratio axis is log-scaled for plotting the entire range of 95% Wald confidence limits. Figure 3 is from Kantarci, K., Lowe, V. J., Lesnick, T. G., Tosakulwong, N., Bailey, K. R., Fields, J. A., et al. (2016). Early postmenopausal transdermal 17beta-estradiol therapy and amyloid-beta deposition. *J Alzheimers Dis*, *53*, 547–556.

## *Discussion*

A participant asked Dr. Miller about the role of the timing hypothesis in her findings. Dr. Miller replied that for women with oophorectomies, it is believed that earlier hormone treatment is better than later treatment for a variety of tissues. However, there is still a question about the ideal duration of treatment. She added that dosing to effect is also important, but it is unclear how to determine the right dose for an individual.

A participant asked about preeclampsia and outcomes; had the researchers looked at the role of the sex of the fetus (i.e., male versus female)? Dr. Miller said they examined whether the sex of the baby had an impact on the outcomes but couldn't find any association.

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A history of preeclampsia places a woman at risk for cardiovascular disease and perhaps accelerated brain damage and cognitive decline as she ages.


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Loss of ovarian function by oophorectomy before the age of natural menopause accelerates accumulation of chronic conditions of aging if women are not treated with estrogen.


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Hormone treatments for women who underwent natural menopause reduced autonomic vascular disturbances, such as hot flashes and night sweats; maintained bone mineral density; and helped sexual function.


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Some premenopausal effects of estrogen and menopausal hormone treatments are modulated by genetic variants. For example, use of transdermal 17β-estradiol for 4 years reduced deposition of β-amyloid in the brains of menopausal women with the ApoE4 variant.

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# SESSION 1: SEX DIFFERENCES IN BRAIN FUNCTION AND BEHAVIOR

***Paul Barrett, Ph.D., NIH/OSC (Moderator)***

## *The Influence of Metabolic Syndrome and Sex on the DNA Methylome in Schizophrenia*

***Kyle J. Burghardt, Pharm.D., Wayne State University, Detroit, MI***

Dr. Kyle Burghardt discussed the role of epigenetics and sex in metabolic syndrome in schizophrenia in an epigenome-wide association study (EWAS). He defined epigenetics as part of the machinery that helps direct how genes are turned on and off. Epigenetics can be influenced by environmental factors, such as diet, lifestyle, and medications. Metabolic syndrome—a constellation of symptoms that includes obesity, dyslipidemia, and hypertension—is about 30% higher in patients with schizophrenia than it is in the general population. In addition, there are sex-specific effects that show an increase of metabolic syndrome in females with schizophrenia.

Dr. Burghardt explained that though atypical antipsychotics are helpful in treating psychiatric symptoms, they are known to cause increased metabolic syndrome when compared with typical antipsychotics. There is therefore a possibility that their use will compromise metabolic or physical health. Dr. Burghardt's study examined whether a differential methylation is occurring that underlies the metabolic syndrome risk in patients with schizophrenia treated with atypical antipsychotics.

Dr. Burghardt conducted a study involving 96 samples (49 males and 47 females), approximately 60% of whom had metabolic syndrome. In males, the top hit was in the CCDC8 gene (chromosome 19). This gene was found to be hypermethylated compared with males without metabolic syndrome. In females, the top hit was in the MAP3K13 gene (chromosome 3), which was found to be hypomethylated compared with females without the syndrome.

An additional study involving 166 subjects attempted to validate these results. The results in males could not be validated. However, the results in females were validated, as three sites in the MAP3K13 gene where hypomethylation occurred were found to be associated with metabolic syndrome.

A literature search found that the CCDC8 gene may be involved in modulating alternative splicing of the insulin receptor, while the MAP3K13 may be a candidate gene in diabetes. Dr. Burghardt said further work is needed in prospective studies to determine any causal correlations. His lab also is studying gene methylation changes in skeletal muscle before and after treatment with atypical antipsychotics.

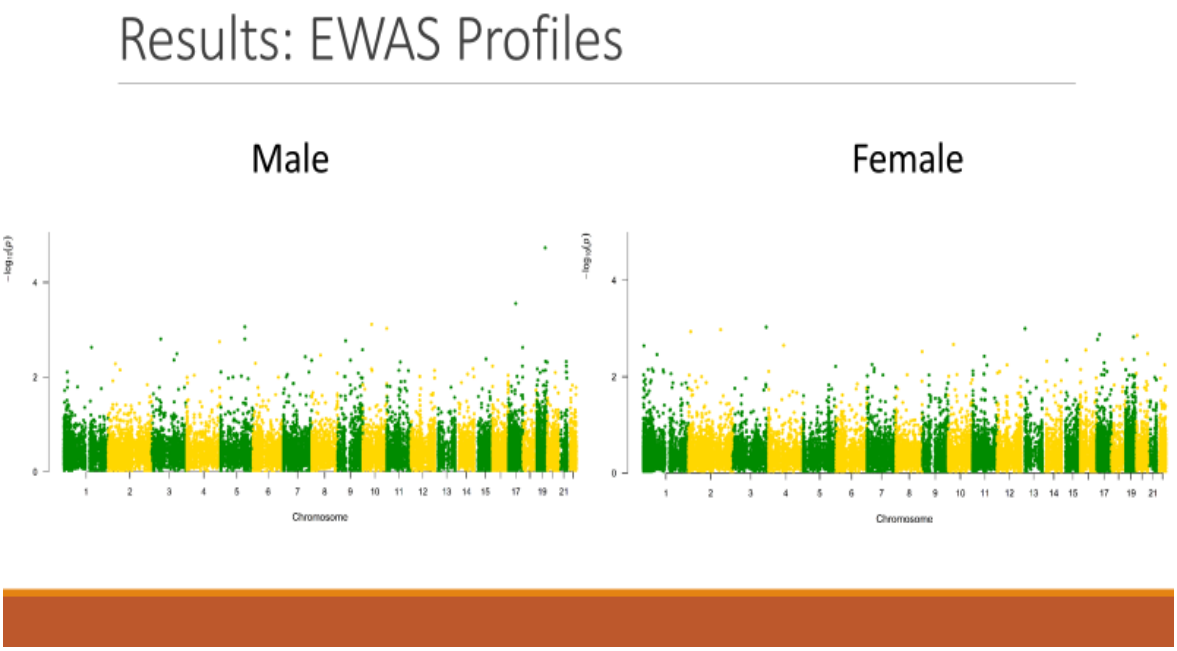


**Figure 1**. 75% power to detect 7.5% difference in DNA methylation at a given probe with a Bonferroni-adjusted p-value.

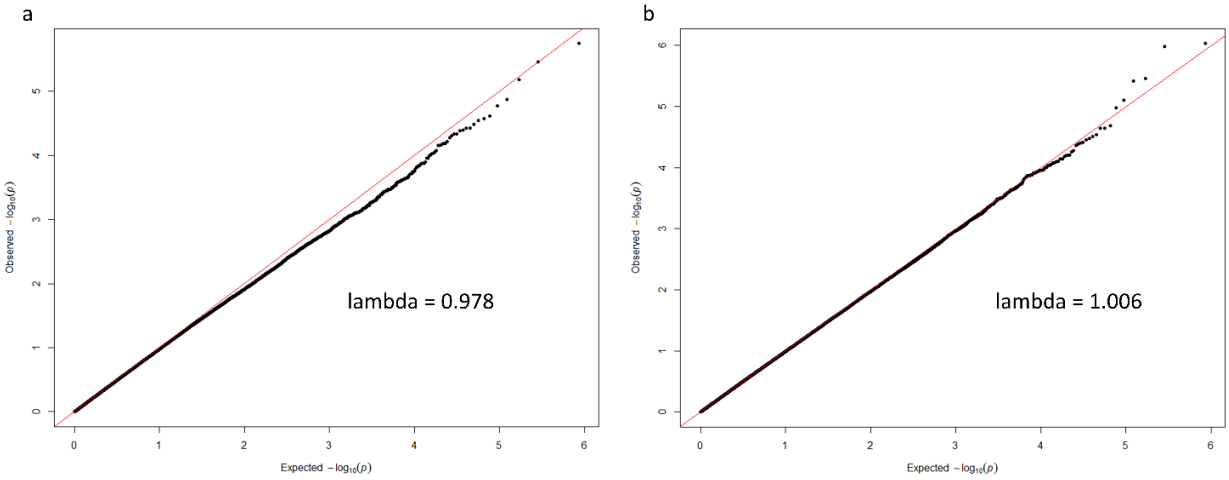


**Figure 2.** QQ plots of sample epigenome-wide analysis. Representative QQ plots for the model assess the association between metabolic syndrome and methylation site using the Illumina Infinium HumanMethylation450 BeadChip for the overall (male and female) populations. Both models included smoking status, antipsychotic type, CD4 T, CD8 T, granulocytes, monocytes, and natural killer cell counts as covariates. Plot a) depicts the QQ plot before performing surrogate variable adjustment (lambda = 0.978), and b) depicts the QQ plot after performing surrogate variable adjustment (lambda = 1.006). The male and female sub-

analyses yielded similar or greater lambda corrections. Including the surrogate variables in the male and female models improved the lambda from 0.889 to 1.001 and 0.893 to 1.021, respectively.

### *Discussion*

- Dr. Miller said a former Building Interdisciplinary Research Careers in Women's Health (BIRCWH) Scholar developed a program that accounts for sex chromosomes within the studies, as well as for exon activation. The free program is available online. She suggested that Dr. Burghardt's lab consider using it to analyze data to determine whether there are any additional genes of importance in his study.
- A participant asked Dr. Burghardt to elaborate on how the MAP3K13 gene may be related to metabolic syndrome. Dr. Burghardt said the connection is mostly with the insulin resistance portion of metabolic syndrome. Proteins within the MAPK/ATK pathway are targeted, which in in turn influences insulin-signaling pathways.
- A participant asked whether more variability was being introduced by incorporating ethnic diversity, seeing as we don't know a great deal about trans-ethnic epigenetic differences. Was ethnicity a confounder in the analysis? Dr. Burghardt replied that in both the EWAS and the validation studies, ethnicity was included as a covariate to adjust the model. He agreed that there is work to be done in epigenetics in terms of race and ethnicity differences. He said participants in the study reflected the demographics of the area in which they were recruited—namely, Ann Arbor, Michigan.

### *Highlights*

- Associations between DNA methylation and antipsychotic-induced metabolic syndrome may occur in a sex-specific manner.
- Future, prospective, and candidate gene studies are needed to understand the interplay of genes, disease, and medication use.

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## *Sex-Specific Metabolic and Epigenetic Changes in Primary Fibroblasts from Patients with Alzheimer's Disease*


***Eugenia Trushina, Ph.D., Mayo Clinic, Rochester, MN***


Dr. Eugenia Trushina's research focuses on the connection between metabolomics and Alzheimer's disease (AD). It is estimated that in the year 2050, 14 million Americans will be afflicted with AD. The hallmarks are senile plaques and neurofibrillary tangles in the brain. However, the mechanisms of AD are not completely understood. Alzheimer's disease is hereditary in approximately 5% of cases (also called familial AD or FAD) and is associated with mutations in the APP, PS1, and PS2 genes. Late-onset

Alzheimer's disease (LOAD), which constitutes the majority of cases (approximately 95%), is not believed to be hereditary, and no specific gene has been associated with it.

Sex differences do exist for AD. The disease disproportionally affects women in both prevalence and severity. Women who test positive for the ApoE4 allele have been found to be at greater risk for developing AD when compared with men with the same allele. On average, women with AD also experience a faster progression of hippocampus atrophy and have more affective symptoms than men; however, they have longer survival time than men. These data suggest that different underlying molecular mechanisms might be in place for each sex.

Studies have shown that perturbation in glucose metabolism and mitochondrial bioenergetics precede the development of AD pathology. Abnormal brain metabolism also has been detected by fluorodeoxyglucose positron emission tomography (FDG-PET) imaging in patients with mild cognitive impairment, suggesting that mitochondria and altered brain metabolism could be involved in early disease mechanisms. Because the development of the disease can begin many years before the manifestation of clinical symptoms, understanding pathophysiological changes in AD patients is one avenue of research that could lead to the development of biomarkers to diagnose the disease earlier and help detect its progression.

In 2014, Dr. Trushina and colleagues published an article that examined the metabolic changes in blood plasma and cerebrospinal fluid (CSF) from the same patients with different AD severity. After examining changes in over 150 metabolites, the researchers found various altered canonical pathways, both in plasma and in CSF. The number of pathways impacted increased with disease severity. Energy metabolism, Krebs cycle, mitochondrial function, neurotransmitter and amino acid metabolism, and lipid biosynthesis were some of the pathways impacted in patients with mild cognitive impairment and with AD.

Further metabolomic profiling of brain tissue in amyloid precursor protein (APP)/PS1 mice found that female mice were impacted to a greater extent when compared with males. This led to a hypothesis that the changes may be caused by a different response in the utilization of alternative fuel to maintain function when the brain can no longer utilize glucose. Studies of brain tissue in postmenopausal female mice showed that they were catabolizing myelin in the brain, which led to white tissue degeneration. This phenomenon also is seen in individuals with AD.

Seeing as brain cells of living humans are not used in diagnosis, Dr. Trushina's lab examined changes in fibroblasts. Human skin fibroblasts of patients with familial AD (PS1 mutation), patients with LOAD, and a control group were studied. Results showed that fibroblasts in both males and females with LOAD had reduced glucose utilization. Metabolic changes were more pronounced in males with AD when compared with females. Male fibroblasts had reduced adenosine triphosphate (ATP) production, while females were unaffected. Male fibroblasts also had a high mitochondrial DNA copy number when compared with females.

Investigators concluded that fibroblasts recapitulate metabolic and epigenetic changes detected in the CSF, blood plasma, and post-mortem brain tissue of individuals with AD. Epigenetic changes in fibroblasts in females with AD seem to indicate a different disease mechanism in females. In addition, fibroblasts in humans with AD in general could represent a valuable model for drug discovery and the evaluation of the molecular mechanisms of AD.

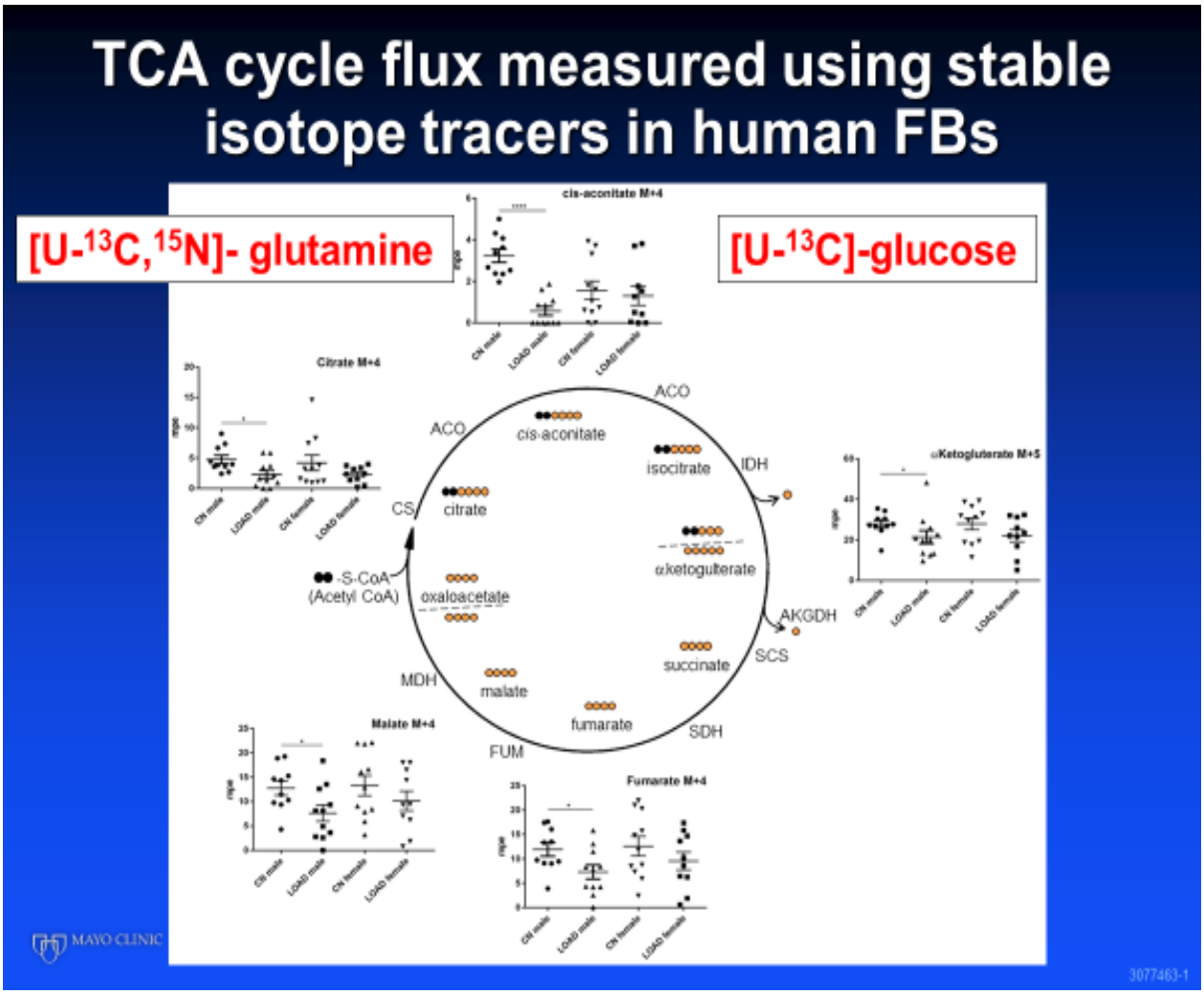


**Figure 1.** Tricarboxylic acid (TCA) cycle flux is significantly perturbed in fibroblasts from males with AD. Skin fibroblasts from cognitively normal males and females and patients with AD were incubated with glutamine-free media supplemented with uniformly labeled glutamine ([$^{13}C_5$, $^{15}N_2$]-glutamine) for 24 hours. Cells were fixed and harvested in 80% methanol. The distribution of uniformly labeled glutamine was determined by GC–MS analysis. Each data point represents a unique patient fibroblast. Black circles represent non-labeled carbon ($^{12}C$), and orange indicates incorporation of the $^{13}C$ isotope. Significance was determined using a t-test. Abbreviations: CN, control; LOAD, late-onset Alzheimer's disease; ACO, aconitase; IDH, isocitrate dehydrogenase; AKGDH, alpha-ketoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; SDH, succinic dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; CS, citrate synthase.

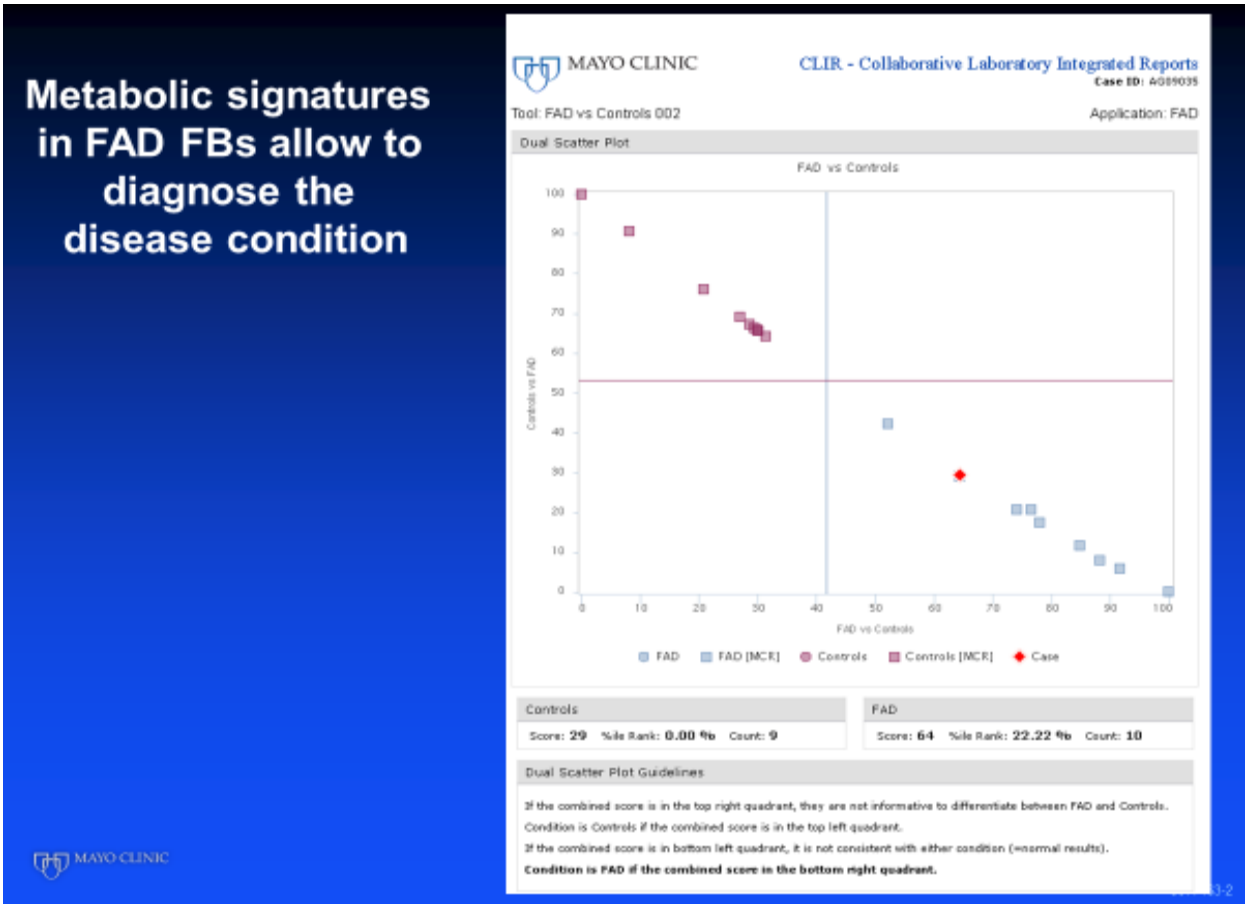


**Figure 2**. Changes in the TCA cycle metabolites in human skin fibroblasts could discriminate between AD patients and cognitively normal individuals. A dual scatter plot is shown, generated using Collaborative Laboratory Integrated Reports (CLIR) software developed at the Mayo Clinic for FAD patients (blue rhombs) and control individuals (purple rhombs) in the training set. The red rhomb represents the FAD patient from the test set.

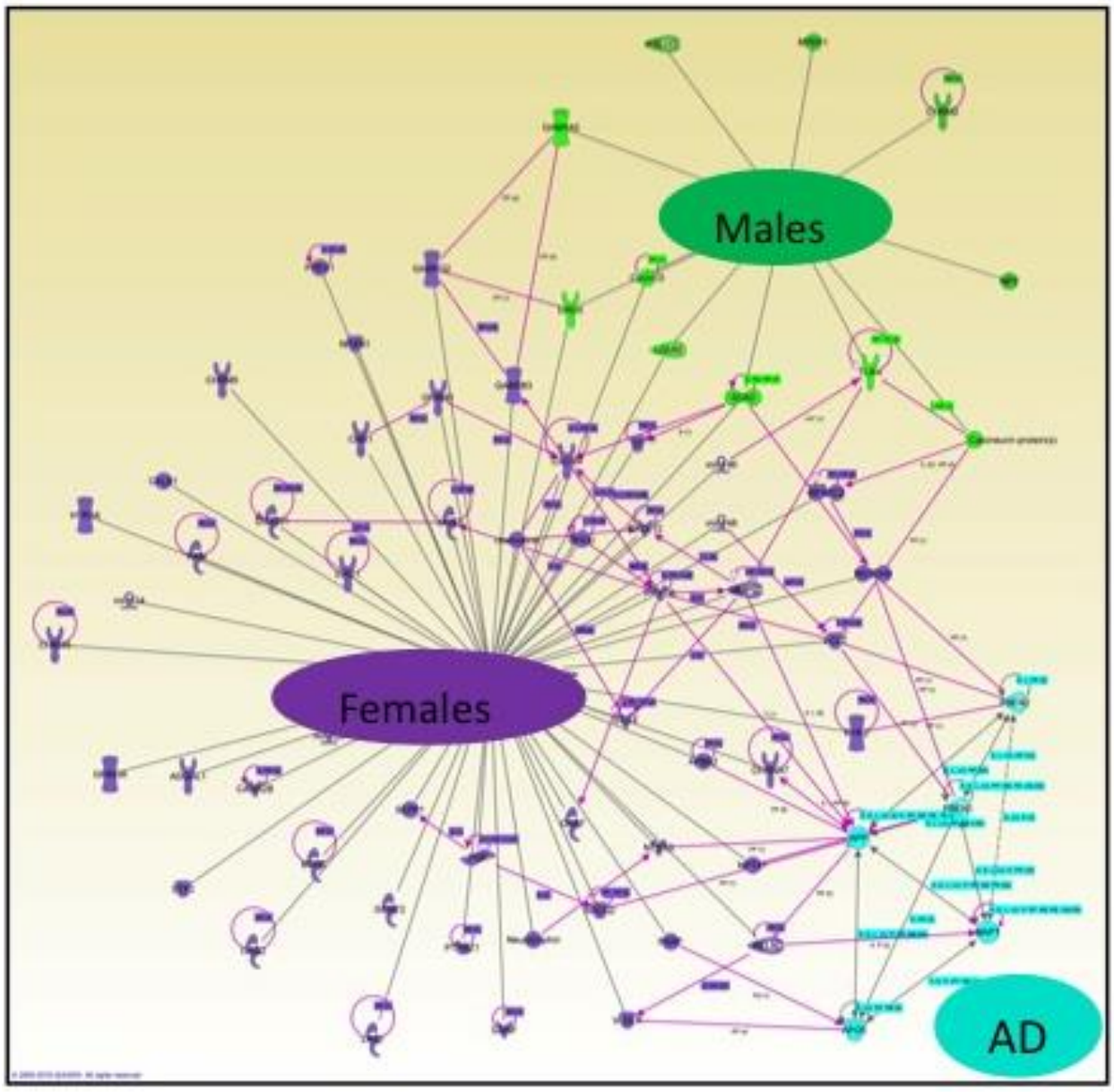


**Figure 3.** Differentially methylated genes in fibroblasts from patients with LOAD are directly linked to AD genetic factors. DNA methylation profiles in skin fibroblasts from LOAD patients were determined using reduced-representation bisulfite sequencing (RRBS). Differentially methylated genes were identified as having > 10% mean methylation difference between LOAD and control cells and a q-value < 0.01. Significantly altered genes in females (purple) and males (green) are shown. Interaction among differentially methylated genes and known familial AD genes is shown. Interaction networks were generated using Ingenuity Pathway Analysis software.

## *Highlights*

- Metabolic changes are associated with the development and progression of Alzheimer's disease. These changes could be detected in the CSF, plasma, and skin fibroblasts using a metabolomics approach.

- Metabolic changes differ between sexes in fibroblasts from control individuals and patients with Alzheimer's disease.
- Metabolic changes are disease-specific.
- Metabolic signatures could be used to identify molecular mechanisms underlying Alzheimer's disease for diagnosis, prognosis, and monitoring therapeutic efficacy.

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## *Sex Differences in Brain–Gut Interactions*


***Emeran A. Mayer, M.D., Ph.D.; University of California, Los Angeles; Los Angeles, CA***


The UCLA Center for Neurovisceral Sciences and Women's Health has been a Specialized Center of Research (SCOR) grantee since 2002. During that time, the center has carried out a series of studies involving sex differences in behavioral and brain responses in patients with chronic visceral pain and, more recently, in individuals with obesity and altered eating behaviors. Dr. Mayer described several of these studies and their findings.

The brain–gut–microbiome axis consists of the interaction between the brain connectome and the gut connectome (i.e., immune, neuronal, and neuroendocrine cells). The gut microbes play an important yet incompletely understood role in these interactions. Putative brain–gut–microbiome diseases and disorders include functional gastrointestinal (GI) disorders such as irritable bowel syndrome (IBS), as well as chronic visceral pain, obesity, and disorders of mood and affect. These disorders are known to have a sex bias in vulnerability and health care–seeking behavior.

Dr. Mayer explained that women are more likely to be affected by most chronic pain conditions and that women are significantly overrepresented in individuals seeking medical help for their symptoms. There also is a female predominance in chronic overlapping pain conditions; women having one chronic pain condition have a higher risk of having another pain condition than men. Yet few neuroimaging studies have addressed these sex and gender differences. A literature review identified 412 studies related to chronic pain and neuroimaging. Of these, only 15 manuscripts were identified as sex differences studies. Similarly, a co-citation network analysis of chronic pain neuroimaging studies with a focus on treatment by sex showed that most fibromyalgia studies are single-sex studies. Few fMRI studies in obesity research have addressed SABV.

Dr. Mayer provided examples of studies of patients with chronic visceral pain published by the UCLA SCOR that demonstrated significant sex-related differences in brain architecture and function, as well as in the relationship of these brain findings to behavioral and clinical measures. He explained that these sex differences exist in the altered morphology, connectivity, and response to pain in patients with chronic pain. For example, there are more prominent primary sensorimotor structural and functional alterations in women, greater insula reactivity in men, and inconsistent differences in emotional-arousal system reactivity. When viewed together, this may indicate that different mechanisms in the brain can produce similar behavioral or clinical outcomes in chronic pain subjects.

Another area of research of the UCLA SCOR is sex differences in obesity and eating behaviors. Among American adults, 78.4 million are considered obese. This includes 36.8 million men and 41.6 million women. Though the aggregated data may not show a distinct difference, when examined by ethnicity and sex, there are clear differences in obesity rates between African American men and women, as well as between Mexican American men and women. In both groups, more women are obese compared with men, and among severely obese individuals, women are more prevalent. In addition, there are significant sex differences in eating behaviors (e.g., uncontrolled hedonic eating behavior). Dr. Mayer presented research results showing sex differences in the connectivity of the reward network, as well as in tripartite association networks involving brain connectivity and subjective measures of stress and ingestive behaviors.

Dr. Mayer emphasized that in light of the disappointing results of decades of preclinical research into chronic pain and obesity without consideration of sex-related differences, it is essential to conduct studies of human brain mechanisms and brain–gut–microbiome interactions that have an emphasis on sex differences. Pharmacological and behavioral treatment strategies could take these differences into account to optimize patient outcomes, as men and women might respond differently to interventions.

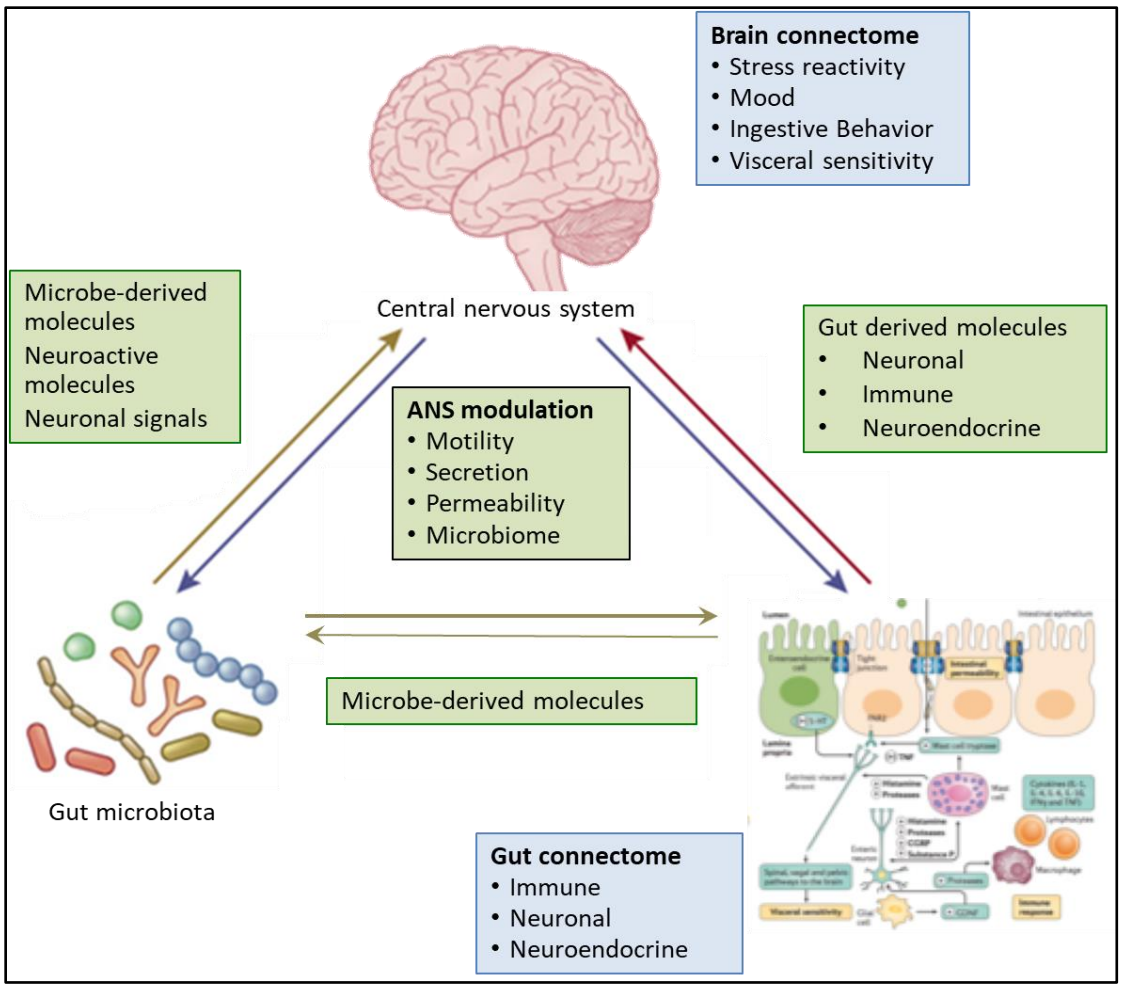


**Figure 1.** The brain–gut–microbiome axis. Modified from Fung, Hsiao et al., *Nature Neuroscience* (2017).

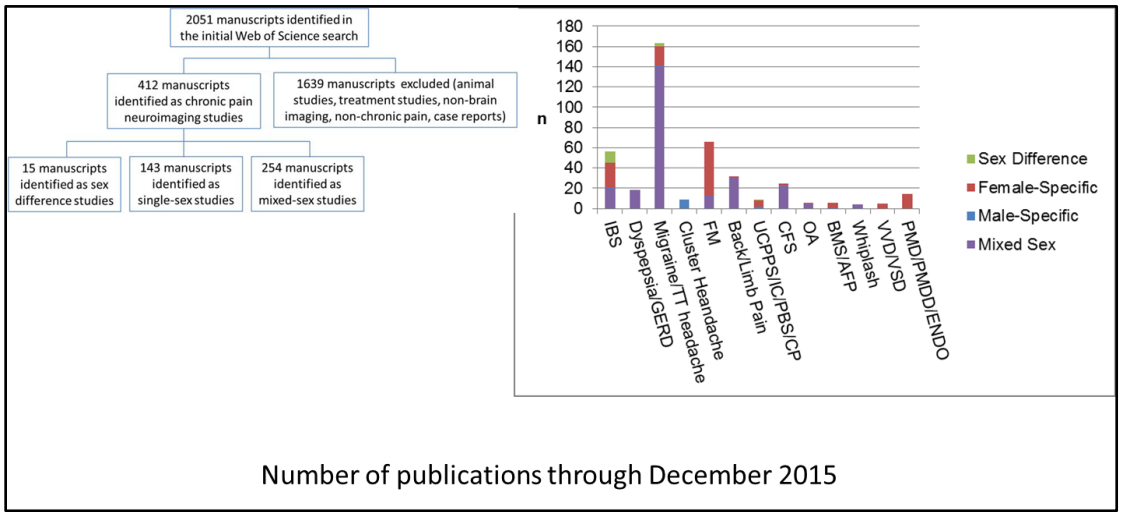


**Figure 2.** Few neuroimaging studies in chronic pain have addressed sex-related differences. Modified from Gupta et al. (2017). *Neuroscience Res.*

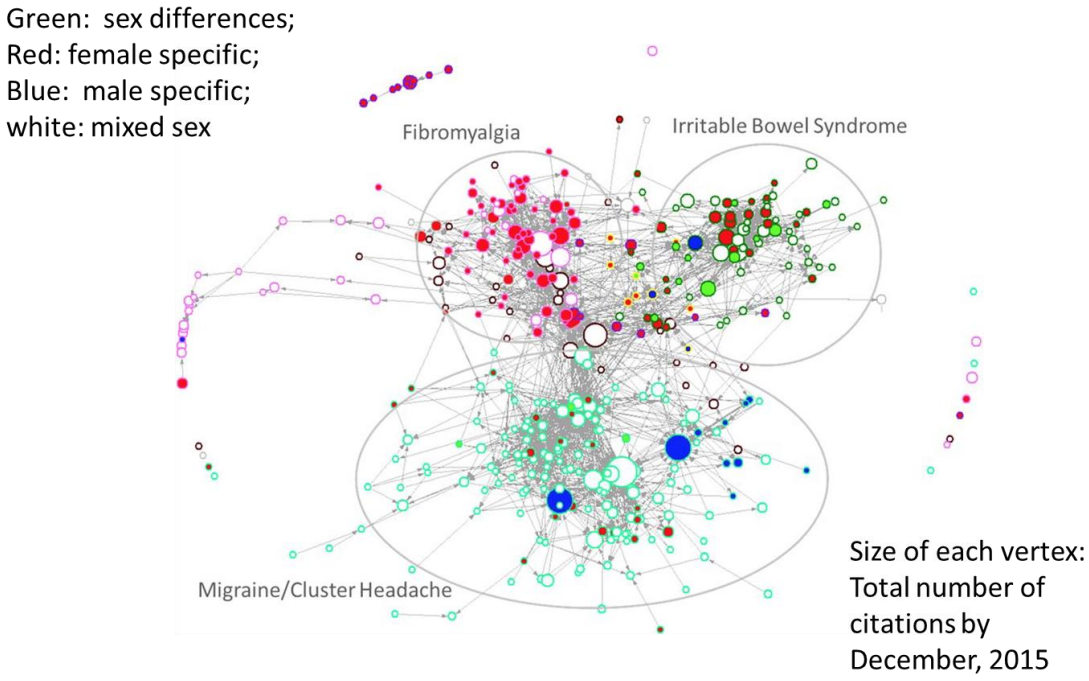


**Figure 3.** Co-citation network analysis of chronic pain neuroimaging studies, with a focus on treatment by sex. Modified from Gupta et al. (2017). *JNR.*

## *Highlights*

- Brain–gut–microbiome interactions play an important role in the regulation of food intake and in chronic visceral pain conditions.
- Epidemiological studies demonstrate that women are more likely to be affected by chronic visceral pain than are men.
- Even though a minority of brain imaging studies in the literature have addressed SABV, these studies demonstrate significant sex-related differences in brain architecture and function and in the relationship of these brain findings with behavioral and clinical measures.
- Pharmacological and behavioral treatment strategies must take these differences into account to optimize outcomes (e.g., personalized medicine).

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## *Incorporating Sex as a Biological Variable in Understanding Brain Development*

***Margaret McCarthy, Ph.D., University of Maryland School of Medicine, Baltimore, MD***

Dr. Margaret McCarthy's presentation highlighted specific sex differences in brain development. She noted that there is a large sex bias in disorders of the nervous system. For example, bulimia, anorexia, multiple sclerosis, post-traumatic stress disorder, anxiety, and depression have a female bias. In contrast, autism, dyslexia, stuttering, attention deficit hyperactivity disorder, and early-onset schizophrenia have a male bias. Neurological processes also can vary by sex. For example, neurulation, myelination, synaptogenesis, and other processes are different in males and females.

Brain differences can be determined by genes, environment, and hormones. The impact of steroid hormones is predominantly seen in the perinatal development period, as well as during puberty. The environment also can impact the brain in ways that we don't fully understand.

Dr. McCarthy's research examines cellular and molecular sex differences in the preoptic area (POA) of the rat model. Her lab developed studies to better understand why the dendritic spine density on the male preoptic neuron is twice the density seen in females. Studies showed that prostaglandin $E_2$ was involved in the mechanism that develops and stabilizes these spines. This led to the study of microglia—the primary immunocompetent cells of the brain—because these cells respond to (and produce) prostaglandins. Microglia cells can take different shapes, some of which produce more prostaglandin than others. Examination of microglia in males found a specifically shaped microglial cell that produces more prostaglandin than the same cell found in females.

Mast cells are another important group of cells involved in sexual differentiation of the brain. They originate in the bone marrow but migrate to all tissues in the body, including the brain. Mast cells act as first responders when injury occurs. When triggered, mast cells can secrete specific molecules that act as mediators. Research has shown that males have more mast cells in the POA during the period when the brain is differentiating, as mast cells secrete histamine, which causes microglia to make more prostaglandins. The prostaglandins, in turn, activate astrocytes, which impact the formation of dendritic spines. Dr. McCarthy's research revealed that there are at least four cell types involved in the synaptogenic process in the POA.

Dr. McCarthy's lab is also studying microglia in the amygdala. Her research has found more phagocytic microglia cells in males compared with females during the first 4 days after birth.

Dr. McCarthy closed her presentation by proposing that the male brain is neither unitarily male nor female. Instead, at a fundamental biological level, the brain is like a mosaic. This means that females may have some masculinized regions and that males may have some regions that are feminized. There also are many regions that are the same in both males and females.

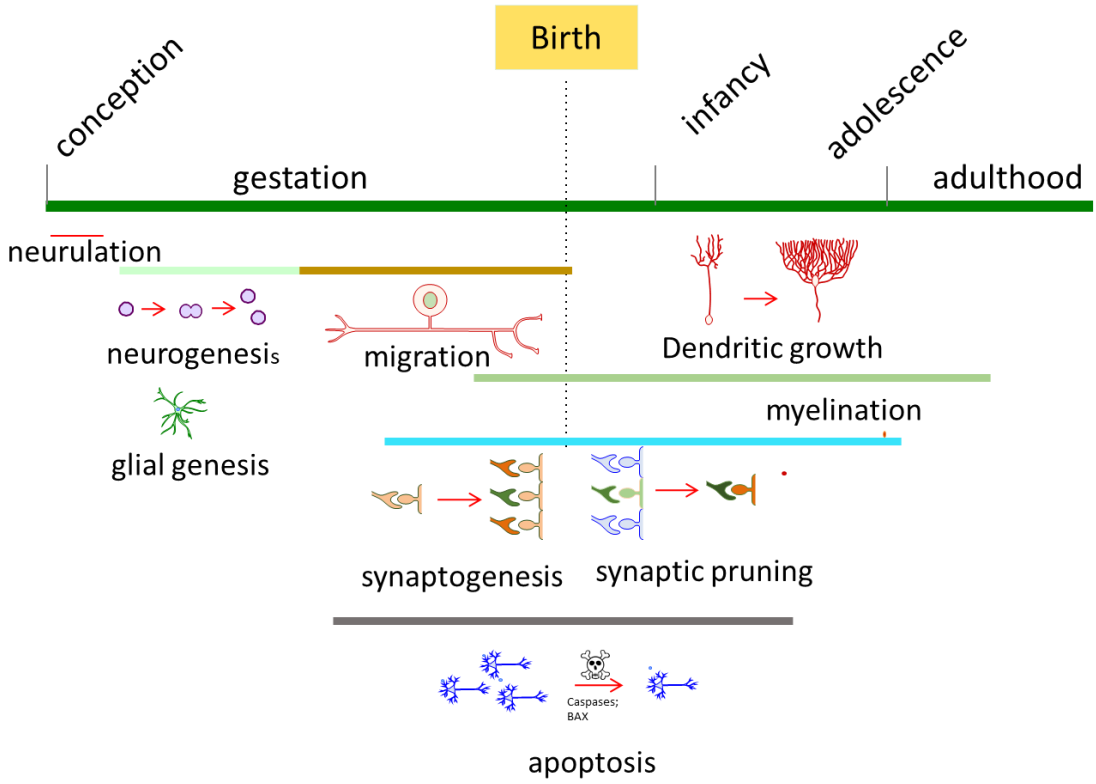


**Figure 1.** Neuroanatomical endpoints that differ in males and females. There are multiple sex differences broadly distributed throughout the brain at the macro and micro levels. Mechanistic studies reveal that every process essential to brain development differs in males and females in some region in some way. This begins with the formation of the neural tube (neurulation), which is impacted by whether an embryo is XX versus XY, and ends with the final maturation of dendrites, axonal projections, and synaptic densities. Both the genesis of cells and naturally occurring cell death (apoptosis) are also important mechanisms by which the size and structure of brain regions can be modified in males versus females. Most of these changes are the consequence of elevated testosterone and its metabolite, estradiol, in the brains of developing males, which differentiates them from females.

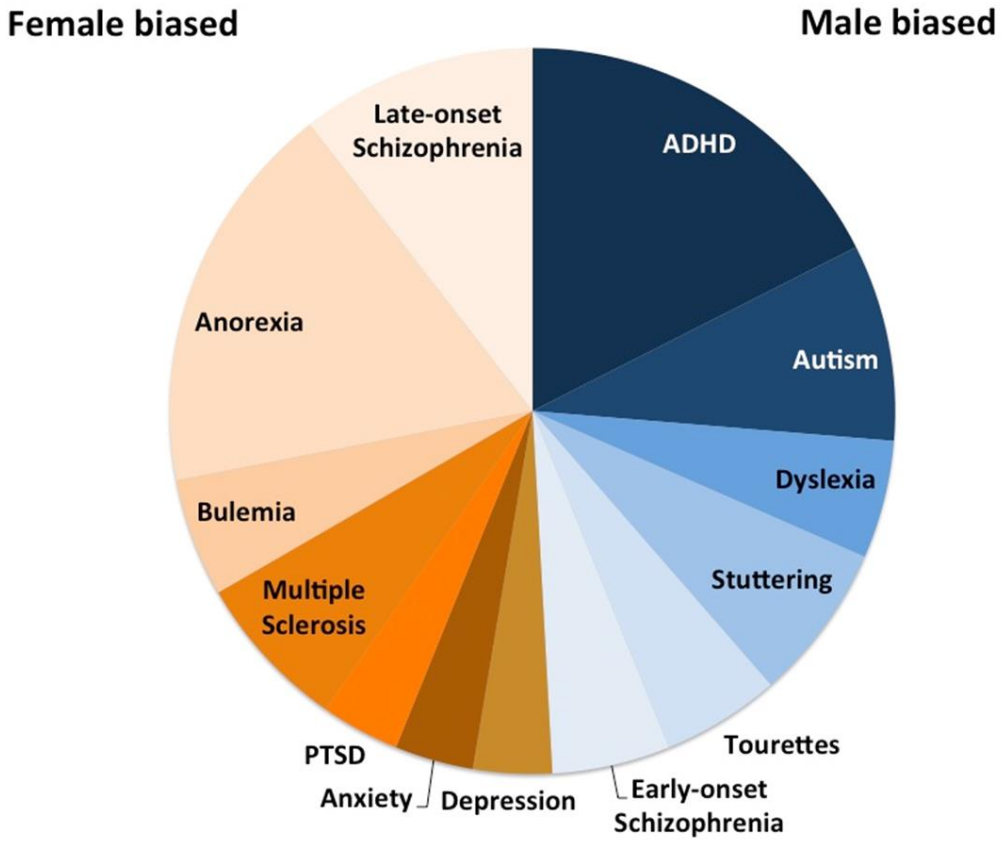


**Figure 2.** Gender bias in neuropsychiatric and neurological disorders. Knowing how the brain develops normally in males versus females is fundamental to understanding the sources of dysregulation that lead to impairments in brain function. Without exception, there is a gender bias in the frequency, symptomology, severity, and/or age of onset of neuropsychiatric and neurological disorders. Those with origins in early development are usually biased toward males; those that more frequently manifest post-puberty are dominant in females. The size of each wedge in this pie chart reflects the magnitude of the reported sex difference on at least one of the above-mentioned parameters.

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# SESSION 2: HOW SEX IMPACTS OUR INTERACTION WITH EXTERNAL INFLUENCES

***Jennifer L. Troyer, Ph.D., National Human Genome Research Institute (NHGRI) (Moderator)***

## *Sex Stratification for Obesity and Related Traits in African Populations—H3Africa Wits-INDEPTH Partnership for Genomic Research Studies*

***Michèle Ramsay, Ph.D., University of the Witwatersrand, Johannesburg, South Africa***

Dr. Michèle Ramsay's presentation focused on the influence of sex in cardiometabolic disease. Differences in obesity can be relative to a specific country or region, socioeconomic status, ethnicity, environment, and/or sex. In many high-income countries, the differences in obesity between males and females are small, but in low- and middle-income countries, differences can often be quite large.

The H3Africa Wits-INDEPTH Partnership for Genomic Research Studies (AWI-Gen) involved approximately 12,000 adults ages 40 to 60 in six African regions: Nairobi (Kenya), Nanoro (Burkina Faso), Navrongo (Ghana), and Dikgale, Agincourt, and Soweto (South Africa). Obesity changes in Africa were found to have a marked sex bias. In South Africa, the prevalence of obesity in women in the three regions ranged from approximately 42% to 67%. In Nairobi, the prevalence in women was 32%. However, in Navrongo and Nanoro, the prevalence in women was approximately 4% and 2%, respectively. The prevalence of obesity in men was strikingly different, ranging from approximately 1% to 18% across the regions.

There also were notable differences in hypertension. The groups studied in South Africa had a notably higher prevalence of hypertension compared with the groups in the other countries. In the two rural South African groups, the prevalence of hypertension was significantly higher in women than in men, but in urban Soweto, there was no sex difference. In urban Nairobi, women were more often affected. In Nanoro, men were more often affected, and Navrongo showed no sex difference. Sex differences also presented in diabetes prevalence. In two South African regions studied and in Nairobi, the prevalence of diabetes was higher in women than in men.

Dr. Ramsay explained that some areas in Africa are undergoing a health and epidemiological transition. For example, compared with other African countries, South Africa is far along in the transition to increased urbanization and lifestyle adaptation. This influences health through the impact of environmental and behavioral changes. The AWI-Gen studies found a correlation between urbanization and increased body mass index (BMI).

Dr. Ramsay also examined obesity relative to a number of demographic, socioeconomic, and behavioral factors, such as age, marital status, education, employment, smoking, tuberculosis, HIV status, diabetes, alcohol consumption, and others. The findings showed a large regional variability by study center, but there also were significant differences between women and men.

The results were still being analyzed, but in Agincourt, South Africa, marriage was associated with an increase in BMI in men, while being single was associated with an increase in BMI in women. In Nanoro, higher socioeconomic status and education level correlated with higher BMI, smoking, and alcohol use; increased age correlated with lower BMI. Dr. Ramsay explained that further work is needed to better

understand genetic risk and gene–environment interactions in susceptibility for cardiometabolic diseases among Africans.

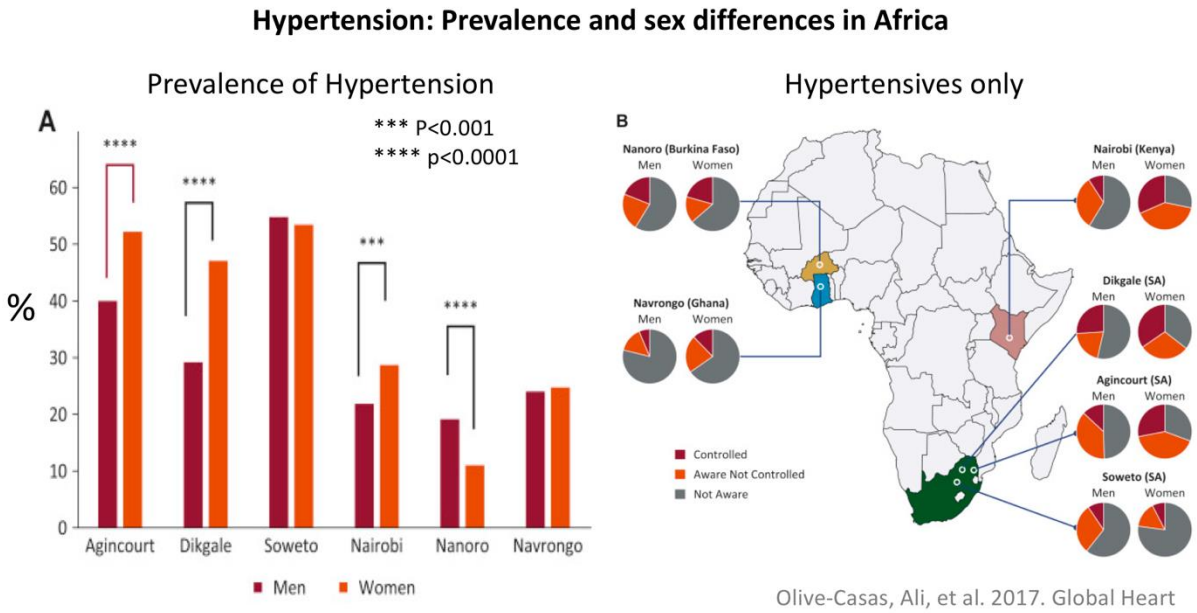


**Figure 1.** Hypertension prevalence and sex differences in Africa.

## *Discussion*

- A participant asked whether the fats used in diets had changed in the regions studied. Dr. Ramsay replied that the diets differ by area. There is a need to more thoroughly investigate fat intake and consumption patterns.

## *Highlights*

- Obesity is a key risk factor for cardiometabolic disease. It is influenced by behavior but also by genetic and other biological factors.
- In low- and middle-income countries, women are disproportionately affected by obesity and its health-related impacts. The reasons for this trend are poorly understood.
- There is considerable regional variation in the prevalence of obesity, hypertension, and diabetes across African regions, with some variability associated with rural-to-urban transitions.
- Public health interventions require a better understanding of the key drivers of the health and epidemiological transition across Africa.

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## *Sex Differences in Pain Management*

***Anne Z. Murphy, Ph.D., Georgia State University, Atlanta, GA***

Dr. Anne Murphy presented her work on sex differences in morphine analgesia. In the past, several rodent studies were conducted to examine sex differences in pain alleviation using morphine, and they reported mixed results. These studies usually used acute pain assays, for which morphine is not typically prescribed and which made results difficult to interpret. Human studies on the self-administration of opioids (i.e., patient-controlled morphine pumps) for post-surgical pain showed that males self-administer morphine more frequently than females. For a long time, this was interpreted as morphine's having greater effectiveness in females than in males. However, it is important to remember that consumption is not necessarily equivalent to analgesia. A 2001 study showed that the negative side effects induced by morphine consumption (e.g., headache, dysphoria, vomiting) are exacerbated in females. This may explain why females self-administer less morphine than males for post-surgical pain relief. Morphine may not be more efficacious; females may be trying to avoid negative side effects.

Dr. Murphy's lab uses a chronic inflammatory pain model in which a rat paw is injected with the bacterial agent complete Freund's adjuvant (CFA). Studies using this pain assay have shown that females require twice the amount of morphine than males to produce comparable levels of analgesia. (See Figure 1A.) These differences in morphine analgesia were not caused by sex differences in baseline pain sensitivity, the hyperalgesia produced by the CFA, or the pharmacokinetics of morphine. Also, no estrous cycle effects were detected on any of the parameters studied. This led researchers to hypothesize that there might be a difference in how morphine acts within the central nervous system in males versus females. Though several mechanisms may contribute to sex-based differences in morphine analgesia, Dr. Murphy's lab chose to focus on the μ-opioid receptor, which is the preferred receptor for morphine. Her lab studied the midbrain periaqueductal gray (PAG), which is a critical brain region for the analgesic effects of opiates.

Research from the Murphy lab showed that males have a greater expression of μ-opioid receptors in the PAG when compared with females. (See Figure 1B.) Consistent with this finding, they learned that

injecting morphine directly into the PAG produced greater analgesia in males. (See Figure 1C.) A greater expression of μ-opioid receptors also was observed in the parabrachial nucleus in males, which is involved in visceral pain. This may be a contributing factor in female visceral pain disorders.

Recent studies have shown that microglia, the resident immune cells of the brain, attenuate morphine action via toll-like receptor 4 (TRL4), a single membrane-spanning receptor found on the surface of microglia. Activation of this receptor initiates the release of proinflammatory cytokines (IL-1β, IL-6, TNF). Immunohistochemical analysis showed high levels of TLR4 expression within the PAG, which may drive some of the observed sex differences in opiate analgesia.

Dr. Murphy's lab found a higher number of activated microglia in the PAG of females compared with males. (See Figure 2A.) Interestingly, the greater the number of activated microglia, the higher the effective morphine dose. (See Figure 2B.) Blocking TRL4 activity in the PAG in females potentiated morphine's ability to produce an analgesic response, effectively cutting in half the effective dose in females compared with males. (See Figure 2C.)

This shows that there are indeed sex differences in both PAG μ-opioid receptor expression and the activation profile of microglia. In the clinical setting, this may indicate that using drugs to inhibit TLR4 could be used as a morphine adjuvant to help potentiate the effectiveness of morphine in females.

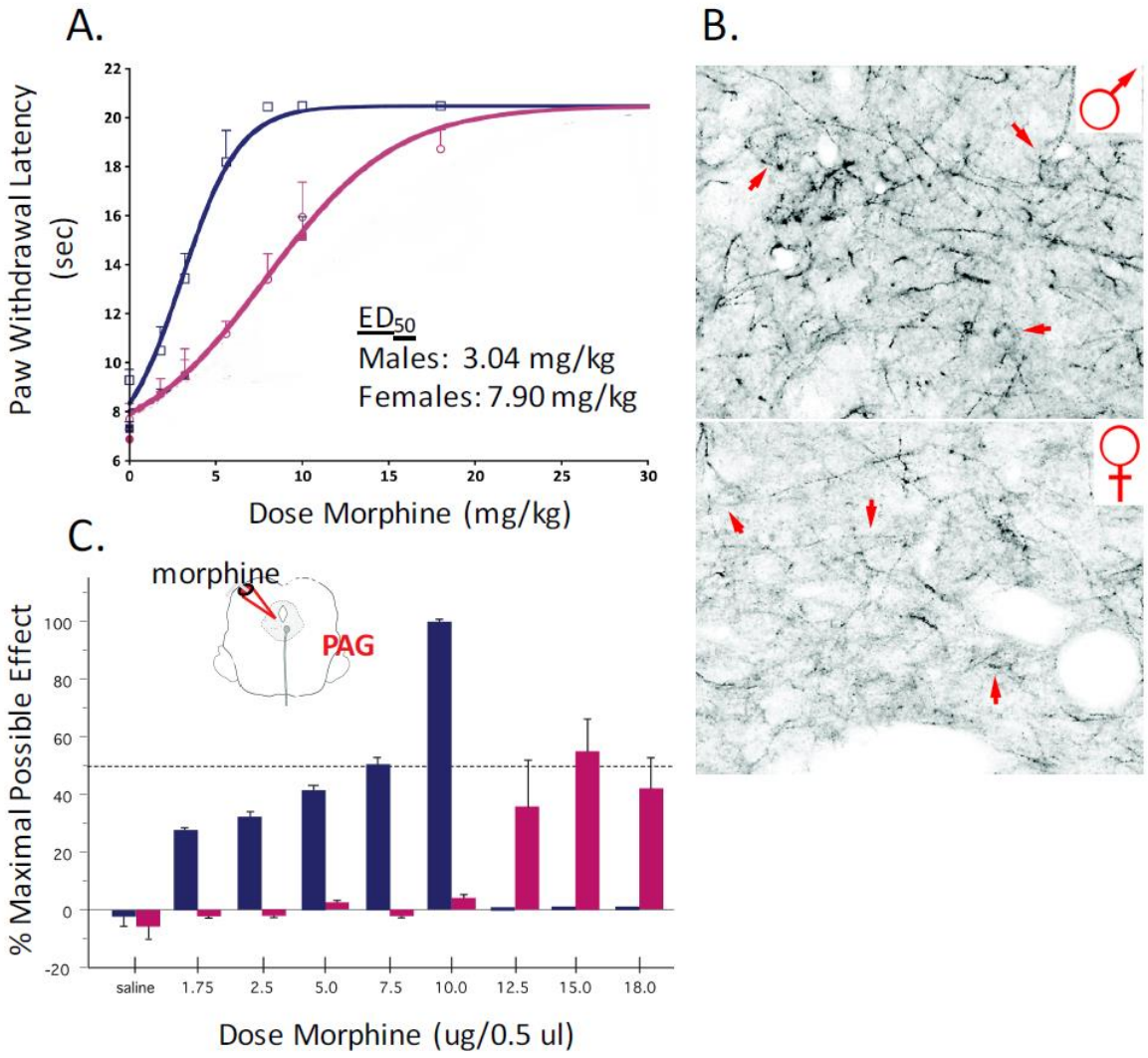


**Figure 1.** Sex differences were found in morphine analgesia. (A) Male (blue) and female (pink) rats received cumulative doses of morphine 24 hours after intraplanar CFA to induce persistent inflammatory pain. Animals were administered morphine every 20 minutes, and the paw withdrawal latency (PWL) in response to a noxious thermal stimulus was recorded. (B) Immunohistochemistry was used to examine the density of μ-opioid receptor (MOR) expression in the midbrain periaqueductal gray (PAG). Males had significantly higher levels of MOR expression than females. (C) Intra-PAG administration of morphine produced greater analgesia in male rats compared with females at all doses tested. Paw withdrawal latencies were converted to % maximum possible response (20 seconds PWL). From Loyd et al., 2009.

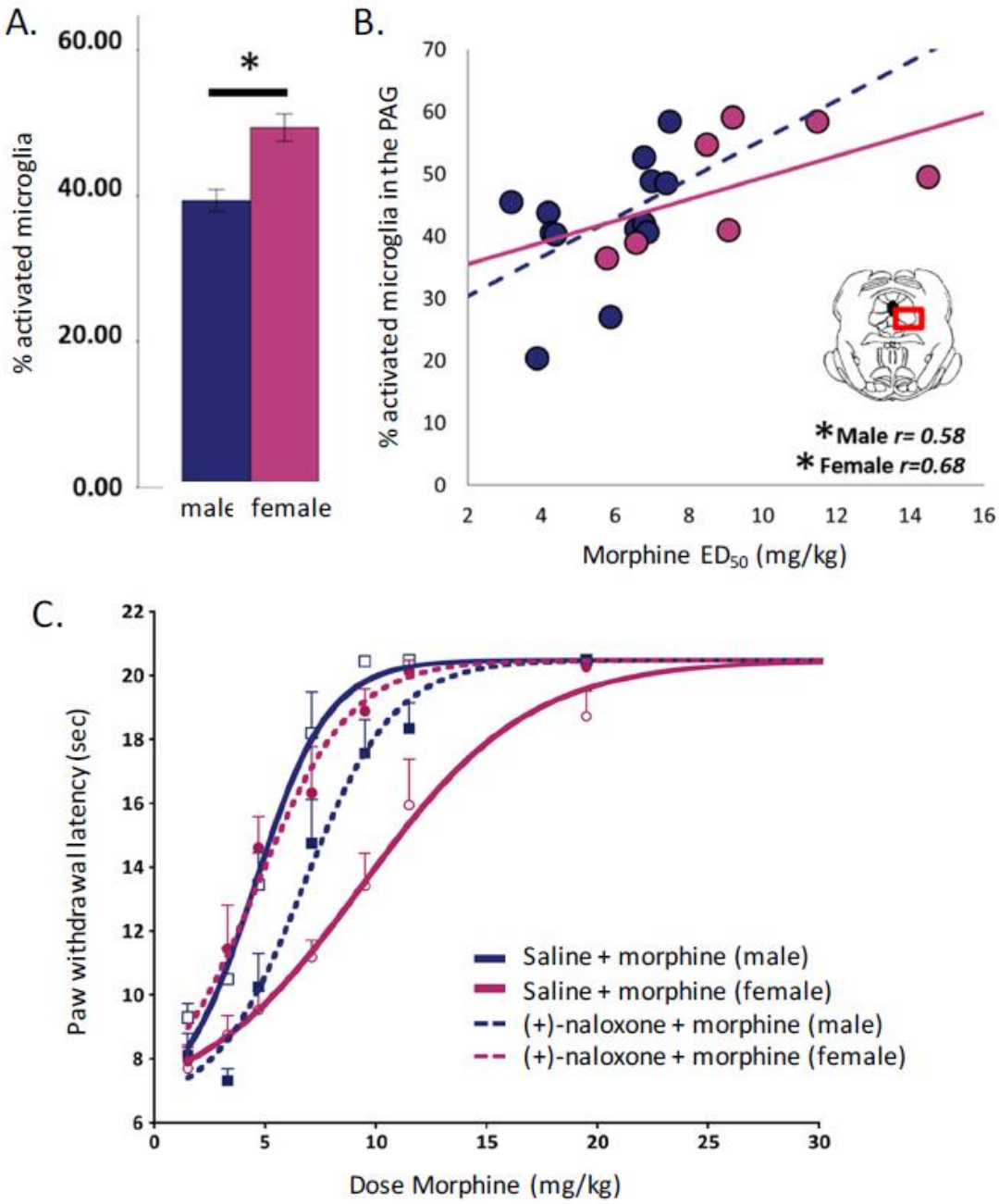


**Figure 2:** Role of the immune system in the sexually dimorphic effects of morphine. (A) PAG microglia show a more activated phenotype in females than in males. Microglia were labeled immunohistochemically using the Iba1 antibody. (B) 50% effective dose of morphine ($ED_{50}$) was significantly correlated with the % of activated microglia in both males and females. (C) Blockade of microglia activity/TLR4 signaling with (+)-naloxone (5.0 μg/0.5 μl; intra-PAG) resulted in a significant 2.5-fold reduction in the morphine $ED_{50}$ dose in females (7.9 to 3.16 mg/kg), but not males (3.04 to 5.25 mg/kg). From Doyle et al., 2017.

## *Highlights*

- Preclinical studies in rodents demonstrate that morphine is more effective in alleviating persistent inflammatory pain in males versus females.

- Sex differences in μ-opioid receptor expression in the periaqueductal gray, a key brain region for opioid action, contribute to the dimorphic effect of morphine on pain relief.
- Microglia, the resident immune cells of the brain, show a more activated profile in females than in males, and may contribute to the increased prevalence rate of chronic pain disorders observed in females.
- Blockade of glial TLR4 signaling decreases opioid dosing requirements 2.5-fold in females and suggests a new avenue for the development of sex-specific pharmacological treatment strategies for pain management.

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## *Sex Differences in Vaccine-induced Immune Responses*


***Nicole E. Basta, Ph.D., University of Minnesota, Minneapolis, MN***


The research of Dr. Nicole Basta in the field of vaccine epidemiology focuses on three primary aims. The first is to evaluate vaccine effectiveness and provide evidence of the breadth and duration of the protection of vaccine-induced immunity. The second is to investigate biological mechanisms to understand the drivers of heterogeneity in immune response and vaccine effectiveness. The third is to assess the population-level impact of vaccination programs, vaccine uptake, and how vaccine acceptance and access change over time.

Extensive preclinical research has demonstrated sex-specific differences in immune system function and response to natural infection. However, there is mixed evidence on the direction and magnitudes of differences in vaccine-induced immune responses for some vaccines in infants, including influenza, tetanus, and group C *Neisseria meningitidis*. Nonetheless, a recent meta-analysis of several clinical trials uncovered sex differences in meningococcal vaccines. The review found a higher response to meningococcal A, W, and Y vaccines among female infants compared with males. The factors that drive sex differences are important because understanding variations in immune response for new vaccines can inform the design of optimal strategies and suggest the timing and number of doses used.

The new MenAfriVac vaccine is a meningococcal A polysaccharide-tetanus toxoid protein conjugate developed by the Meningitis Vaccine Project. It was first introduced in Mali, Niger, and Burkina Faso in 2010. Since then, mass-vaccination campaigns have targeted all individuals ages 1 to 29, and plans are

underway to introduce the vaccine into the infant vaccination schedule. To date, more than 300 million people have received this vaccine in more than 20 countries.

In 2012, Dr. Basta launched the MenAfriVac Antibody Persistence (MAP) study. It's a prospective, longitudinal study that aims to investigate population-level changes in immunity after the introduction of MenAfriVac. A representative sample of 800 randomly selected residents of Bamako, Mali, ages 1 to 29, who were vaccinated during the MenAfriVac mass-vaccination campaign, were recruited and enrolled.

Serological surveys were conducted 2 years and 3.5 years after vaccination. The primary outcome was MenA-specific serum bacterial antibody titers (rSBA), which are the accepted correlate of protection used for licensing MenA vaccines. The secondary outcome was tetanus toxoid IgG levels, because the vaccine was expected to boost tetanus immunity. Results showed a robust and persistent immune response 2 and 3.5 years after vaccination across all age groups. When data were disaggregated, variations were found by age and sex. In individuals under 18 years of age, females showed higher geometric mean titers when compared with males. However, in individuals ages 18 to 29, males had higher titers. Similar trends persisted 3 years after vaccination.

A number of preclinical studies suggested several mechanisms that could explain these differences. One mechanism being investigated in Dr. Basta's group is the role of nutritional biomarkers in immune response. Preliminary analyses suggest that sex may be an important effect modifier of the relationship between micronutrient levels and immune responses to meningococcal A vaccines.

The MAP study also showed a significant boosting of tetanus toxoid IgG after MenAfriVac administration in both males and females compared with titers measured before vaccination. Heterogeneity in baseline tetanus immunity in the population reflects sex-specific vaccination programs that target women of childbearing ages for tetanus vaccination. Immune responses increased proportionately across all age–sex groups, and evidence of this increase persisted 3.5 years after vaccination. This is an important finding in a region where tetanus and neonatal tetanus are still problems.

Dr. Basta explained that their study was powered to assess only age-specific differences at the outset. She said that to better assess SABV, larger sample sizes and advanced planning for data collection would be needed. She also emphasized the importance of using hypotheses generated and tested in epidemiologic studies to inform basic science studies, and vice versa.

### *Discussion*

- A participant noted that there was a twofold difference in titers between females and males. She asked whether there was a way to model the efficacy of those titer differences in neutralizing the organism. Dr. Basta said that it is unclear whether the differences seen in vaccine-induced immunity are clinically relevant, especially in the context of the African meningitis belt, where there are high pre-vaccination titers because of the circulation of carriage strains. It is unknown whether a higher titer response for females would make a difference with respect to protection. However, it may indicate a slower waning over time and perhaps a longer duration of immunity.
- A participant asked whether there were any data about sex-specific differences according to the adjuvant rather than the primary antigen used. Dr. Basta said she did not know of any studies

that reported sex-specific differences with respect to the adjuvant used in a vaccine. Several studies have looked at reported side effects and found that females experience and report side effects more commonly than males across all age groups.

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## *Sex Differences in the Endocannabinoid System Determine Differences in Juvenile Rough-and-Tumble Play Behavior and Correlating Differences in Phagoptosis by Microglia in the Developing Amygdala*


***Kathryn J. Argue, Ph.D., University of Maryland School of Medicine, Baltimore, MD***


Dr. Kathryn Argue's research focuses on the difference in play behavior between male and female rats and the link between play and the endocannabinoid system. Rough-and-tumble behavior—commonly referred to as "play fighting"—is seen across all mammalian species. This type of behavior is critical for brain maturation in the juvenile period. With rare exceptions, this behavior is more frequent and intense in juvenile males than it is in females.

The endocannabinoid system is implicated in many brain development components, including proliferation, differentiation, migration, and communication among nearly all brain cell types. This system is one of the earliest to be turned on during development, with components seen as early as embryonic day 8. One of the important ligands in the endocannabinoid system and the brain is 2-arachidonoylglycerol (2-AG). This ligand primarily acts on $CB_1$ and $CB_2$ type receptors.

Previous research has shown that the sex difference in rough-and-tumble play is mediated by the medial amygdala in the brain. When the amygdala is dissected on postnatal day 4, males have a higher level of 2-AG when compared with females. If females are injected on the day of birth as well as on the day after birth with testosterone, masculinization is seen in the 2-AG levels, which can be blocked by administering an androgen receptor antagonist along with testosterone.

Dr. Argue injected females with $CB_1$ and $CB_2$ receptor agonists and saw an increase in their play behavior. Injecting males with the same agonists did not show a change in play behavior. However, when males were injected with $CB_1$ and $CB_2$ receptor antagonists, there was a decrease in play behavior (feminization). The same antagonists had no effect in females.

In another study, Dr. Argue found a sex difference in the number of newly proliferated cells in the neonatal amygdala. The number of newly proliferated cells correlated with the number of phagocytic microglia. It also was found that phagoptosis of newborn cells was mediated by both the endocannabinoid system and testosterone. Currently, Dr. Argue is trying to actively inhibit phagocytosis in males to determine whether it can affect social play behavior.

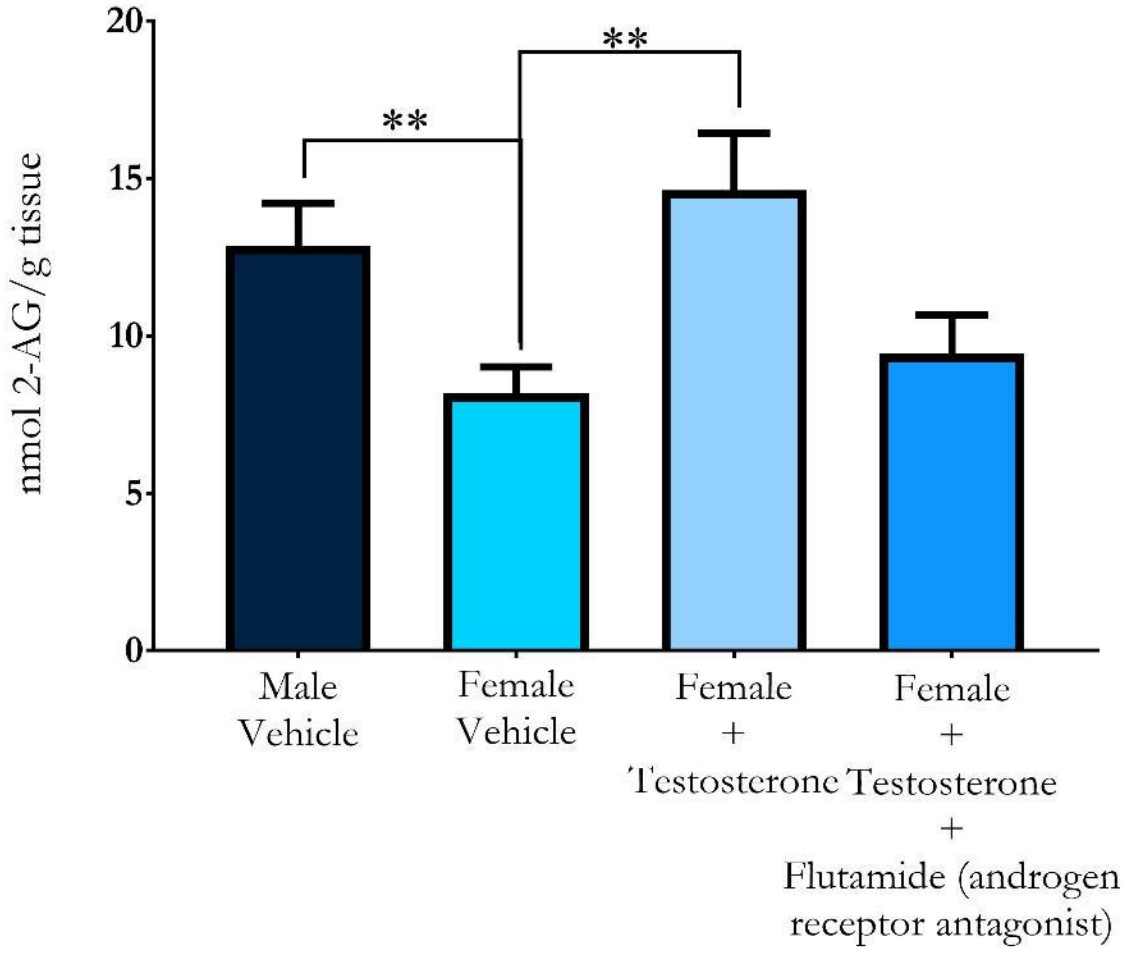


**Figure 1**. Because of higher levels of testosterone, neonatal males at postnatal day 4 have higher levels of the endocannabinoid 2-arachidonoylglycerol (2-AG) in the amygdala.

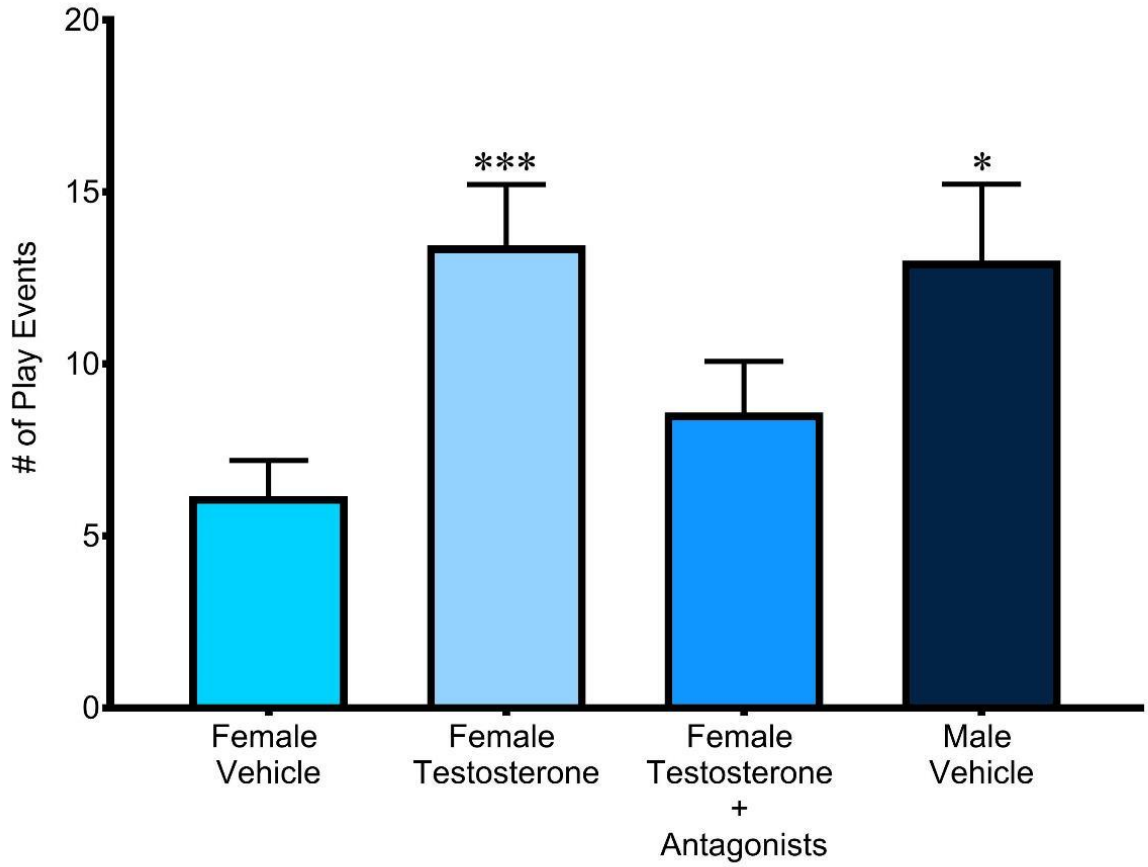


**Figure 2**. Juvenile males have higher levels of rough-and-tumble play relative to females. Neonatal treatment with testosterone can increase play in females, but this effect can be blocked when testosterone is co-administered with endocannabinoid receptor antagonists.

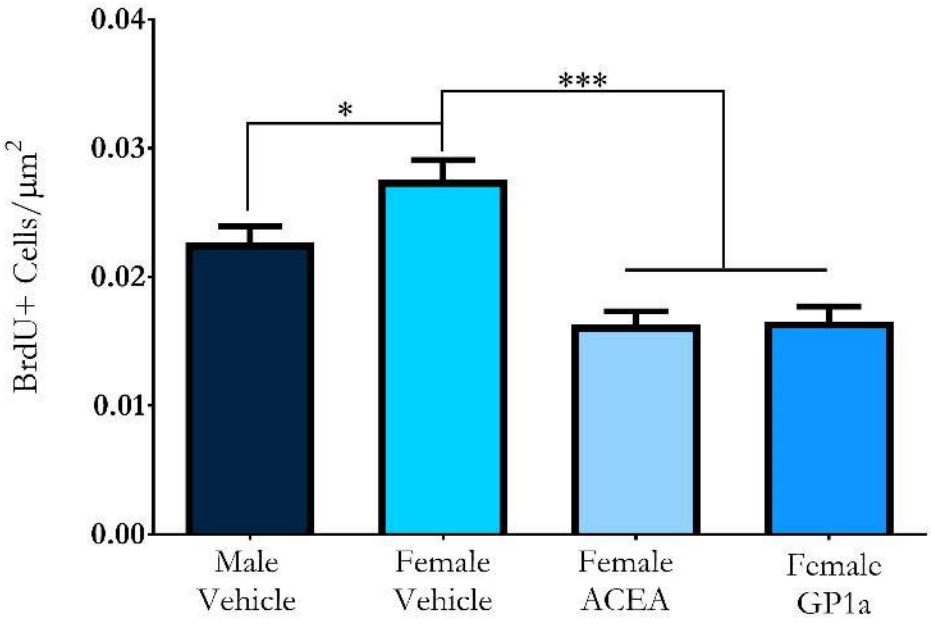


**Figure 3.** There also is a sex difference in the number of newly proliferated cells in the neonatal amygdala that is mediated by the endocannabinoid system, such that mimicking higher levels of 2-AG through administration of endocannabinoid receptor agonists is sufficient to masculinize the number of newly proliferated cells (BrdU+ cells). These data correlate with levels of microglia phagocytosis of newborn cells, suggesting that endocannabinoids act to decrease levels of newborn cells by increasing their engulfment by microglia. The researchers are working on determining the fate of the newborn cells to link the sex differences in neonatal newborn cell number to subsequent juvenile play behavior.

## *Discussion*

- A participant said that in one of the experiments presented, the same response was seen to estrogen, dehydroepiandrosterone (DHEA), and testosterone when used to match the male response. She asked whether they knew why this was the case. Dr. Argue said the increase in 2-AG seems to require action of both testosterone and estradiol. In other words, there seems to be a combined action in the amygdala, such that one needs both testosterone and estradiol for masculinization. As a result, if one gives DHEA, which cannot be aromatized to estradiol, no increase is seen.

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# SESSION 3: SEX DIFFERENCES IN ANIMAL MODELS

***Colin Fletcher, Ph.D., National Human Genome Research Institute (NHGRI) (Moderator)***

## *Sex in Perinatal Brain Damage: Why Can't Boys Be More Like Girls?*

***Anna Penn, M.D., Ph.D., Children's National Medical Center, Washington, DC***

Dr. Anna Penn indicated that extremely preterm males (22–25 weeks) have lower cognitive scores when compared with females. Her presentation focused on how the in utero development period could account for these differences. Understanding the factors involved in neurodevelopment could improve outcomes for preterm babies. Dr. Penn stated that the placenta plays a fundamental role in shaping fetal brain development by contributing to the organization and health of the brain. The placenta is a fetal organ with the same genotype as the fetus.

Dr. Penn's lab used the inflammation/hypoxia mouse model to mimic preterm injury and test the effects of hormone replacement. Understanding the impact of hormones at an early stage is important because preterm babies under 25 weeks have not yet been exposed to a major surge of maternal steroids (estrogen and progesterone); these are traditionally produced later in the pregnancy. Using the hypoxia model, neonatal males and females both showed regional brain volume loss, with males showing a greater percentage of loss. There also was notable myelin loss in male mice. When the mice were adults, the female regional brain volumes recovered somewhat, while the male hippocampal and cerebellar volumes remained reduced.

Researchers targeted allopregnanolone (ALLO), which is a highly neuroactive steroid and a progesterone derivative. The placenta is the main source of ALLO during late gestation. It is easily transferred from the placenta to the fetus to circulation to the brain. ALLO is also nongenomic and acts primarily on the extrasynaptic $GABA_A$ receptor sites to modulate excitatory/inhibitory tone.

Studies were conducted on placental-specific ALLO knockout mice. This model showed a 50% reduction in the production of ALLO in the placenta and a 50% reduction of ALLO in the fetus brain. There was a loss of upper-layer (II/III) neurons in the embryonic cortex that was only significant in males. RNA-seq analysis in these adult mice revealed sex differences in differential gene expression, with more differentially expressed genes identified in male mice that lost placental hormones compared with the changes seen in females. This indicates significant consequences long after the initial insult. Further analysis revealed that the top five diseases and biological functions in the ALLO knockout male mice involved myelination and the activation of microglia. However, in females, the top diseases or biological functions that were impacted involved neurogenesis.

It is not yet clear whether the underlying mechanism of this susceptibility to perinatal brain injury differs between males and females or whether the timing of the developmental events differs between males and females. Determining which of these possibilities has greater importance in the observed sex difference will dictate the treatment to be administered. Researchers also are trying to determine whether placental hormones can be used to protect the developing brain. If this is the case, placental hormones may provide a new therapeutic approach for perinatal brain injury.

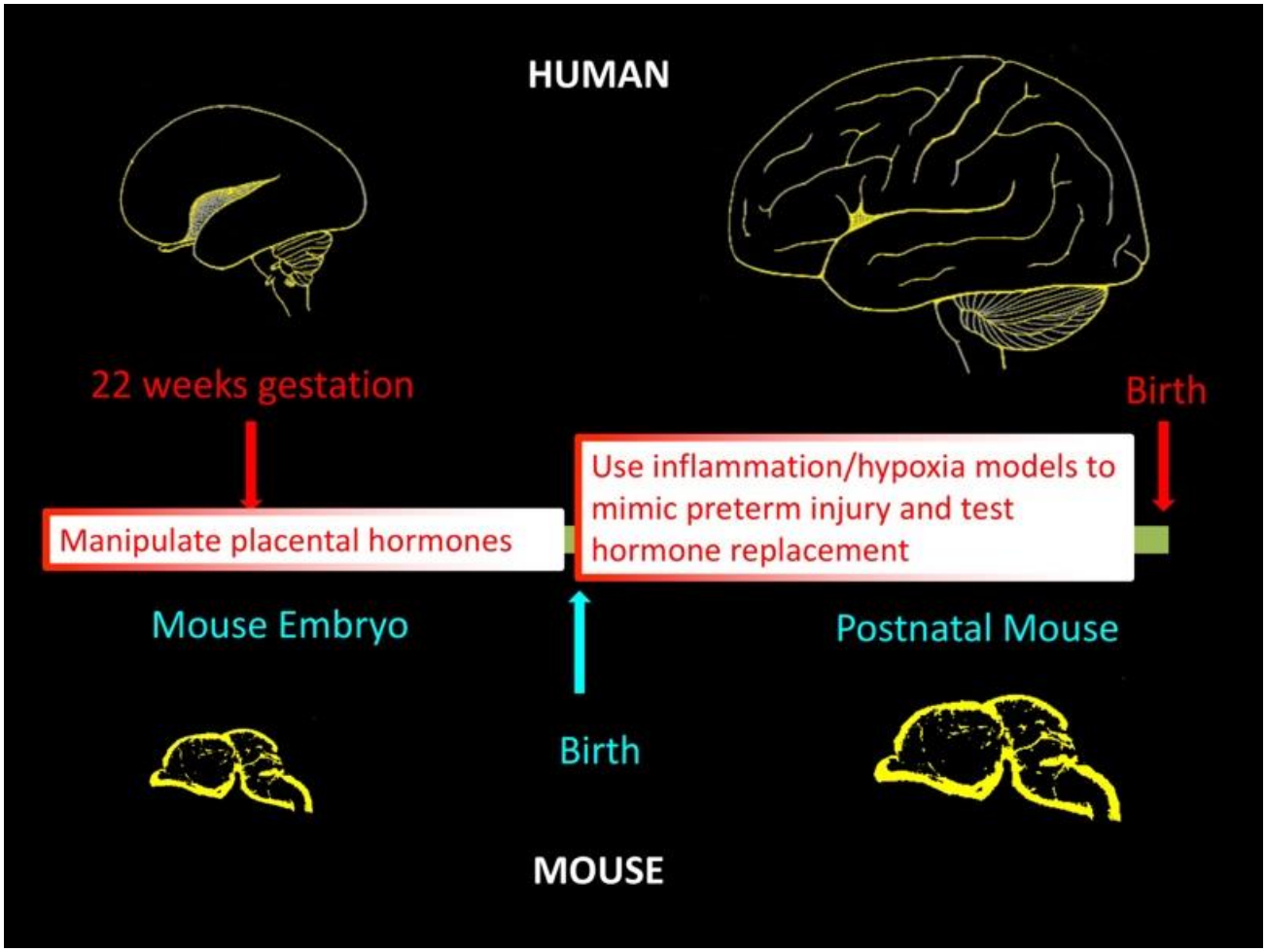


**Figure 1.** Placental hormones were manipulated at 22 weeks gestation, with hormone replacement tested after birth.

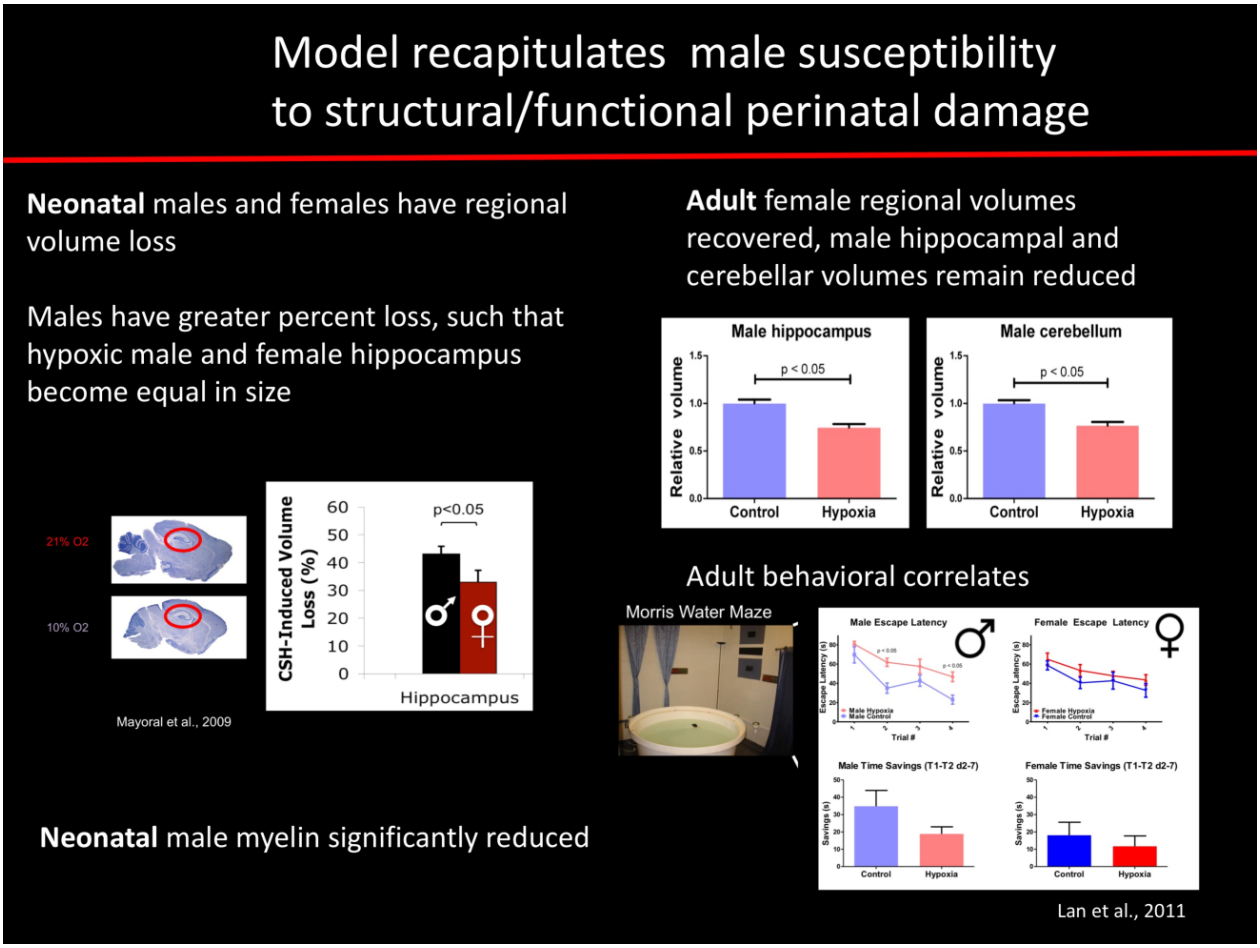


**Figure 2**. The model used recapitulates male susceptibility to structural/functional perinatal damage.

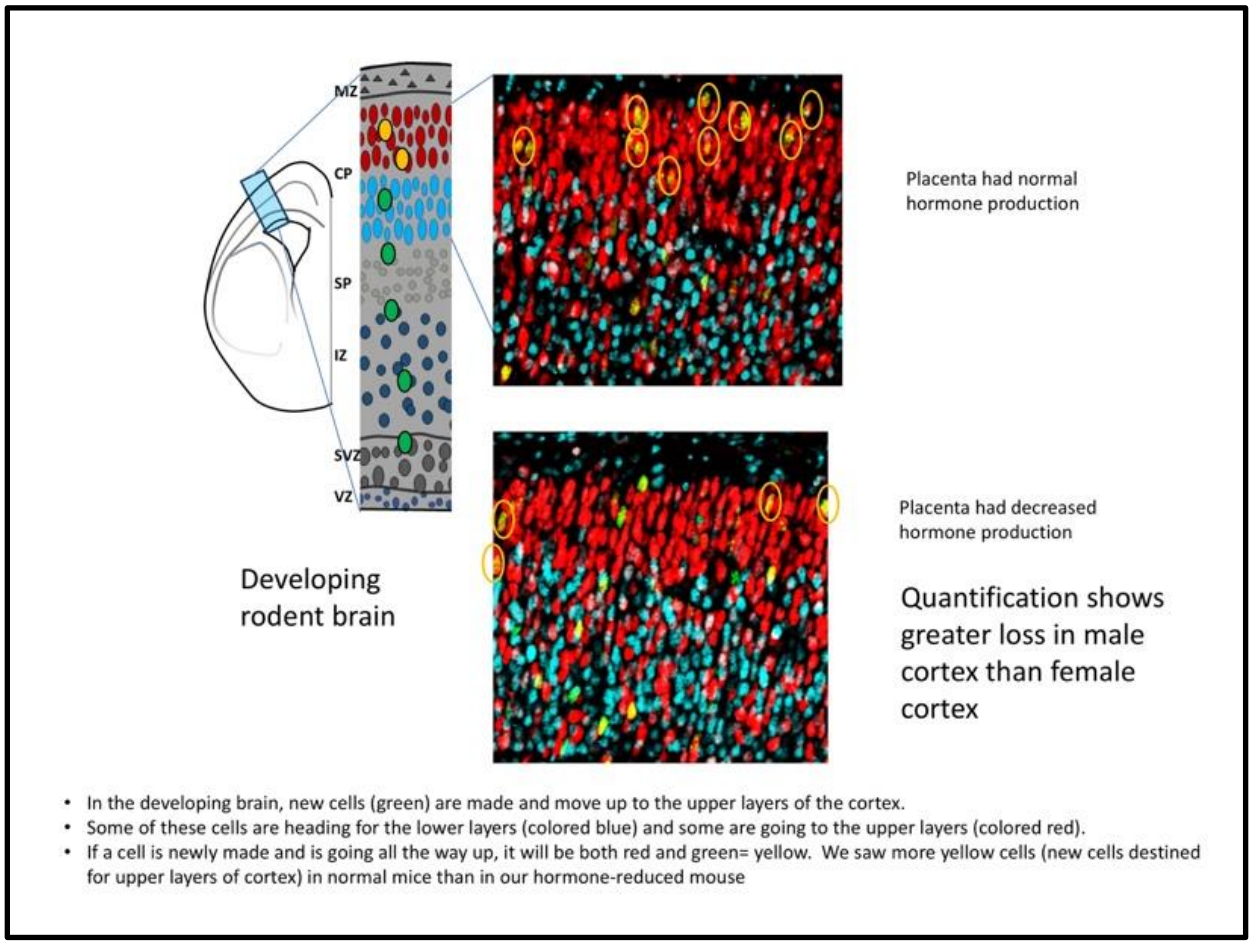


**Figure 3.** Quantification showed greater loss in the male cortex than in the female cortex.

## *Highlights*

- Sex differences exist in the placenta, the brain, and the feto-placental unit as a whole.
- Placental endocrine differences can specifically shape divergent long-term outcomes.
- Preclinical models are beginning to provide mechanistic insights into sex differences that will guide the development of optimal human therapies.

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## *Control of Autoimmunity by Genes, Sex, and the Microbiome*


***Jayne Danska, Ph.D., The Hospital for Sick Children, Toronto, ON***


Dr. Jayne Danska said that sex biases exist in many autoimmune diseases, including Sjögren's syndrome, lupus, autoimmune thyroiditis, myasthenia gravis, rheumatoid arthritis, and multiple sclerosis, all of which have a female bias. Some studies have also shown that the bias in these diseases and conditions increases with age at onset.

Sex effects also can be modifiers of disease and gene activity. For example, in multiple sclerosis, although the number of cases is greater in females than in males, the difficulties of managing men with multiple sclerosis can be far more challenging. Therefore, one must consider an expanded frame of reference with regard to sex effects in disease that goes beyond incidence.

The relative contributions of different mechanisms with regard to autoimmune disease are not fully known. Clearly, there are effects of hormones as well as contributions from X and Y chromosome–linked genes, whose behavior can be epigenetically patterned. There also are differences between males and females with regard to responses to environment, as well as sex differences in epigenetic patterning and regulation of autosomal genes that have nothing to do with sexually differentiated tissues.

Over time, there have been changes in public health approaches in developed nations with regard to the water supply, treatment of milk products with pasteurization, the husbandry of animals for human consumption, an increase in fast-food diets, and the use of xenobiotic drugs, particularly antibiotics. These changes have been associated with changes in the microbial universe that individuals come in contact with, as well as changes in the microbiota.

Studies have shown that the incidence of type 1 diabetes in certain animal models increases in both males and females over time, but a significant sex difference emerges by week 40. However, when this same experiment is done in a germ-free environment, the incidence is nearly the same for both males and females.

Further studies have shown that though there is not a significant sex difference in the gut microbiome composition of non-obese diabetic (NOD) mice at weaning, a sex difference appears over time, with a significant difference being present once the mice reach adulthood. In other words, the difference in

composition of microbes in the guts of male and female mice is insignificant at an early age but pronounced as the mice reach adulthood.

Experiments were conducted to modify the gut microbiome of young females through microbial transfers. By using oral gavage at 3 weeks of age, researchers changed the gut microbiome of NOD female mice from that of adult females to that of adult males. This alteration of the gut microbiome composition was found to persist at 14 weeks of age. Young females who had their gut microbiome changed to a male microbiome had a much lower incidence of type 1 diabetes (nearly 70% reduction in incidence). In other words, the male microbiome composition conferred protection from type 1 diabetes.

In addition, the females that received male gut microbes showed a higher concentration of testosterone and precursors of testosterone in their blood serum when compared with control females. This indicates that gut microbes affect testosterone levels in males as well.

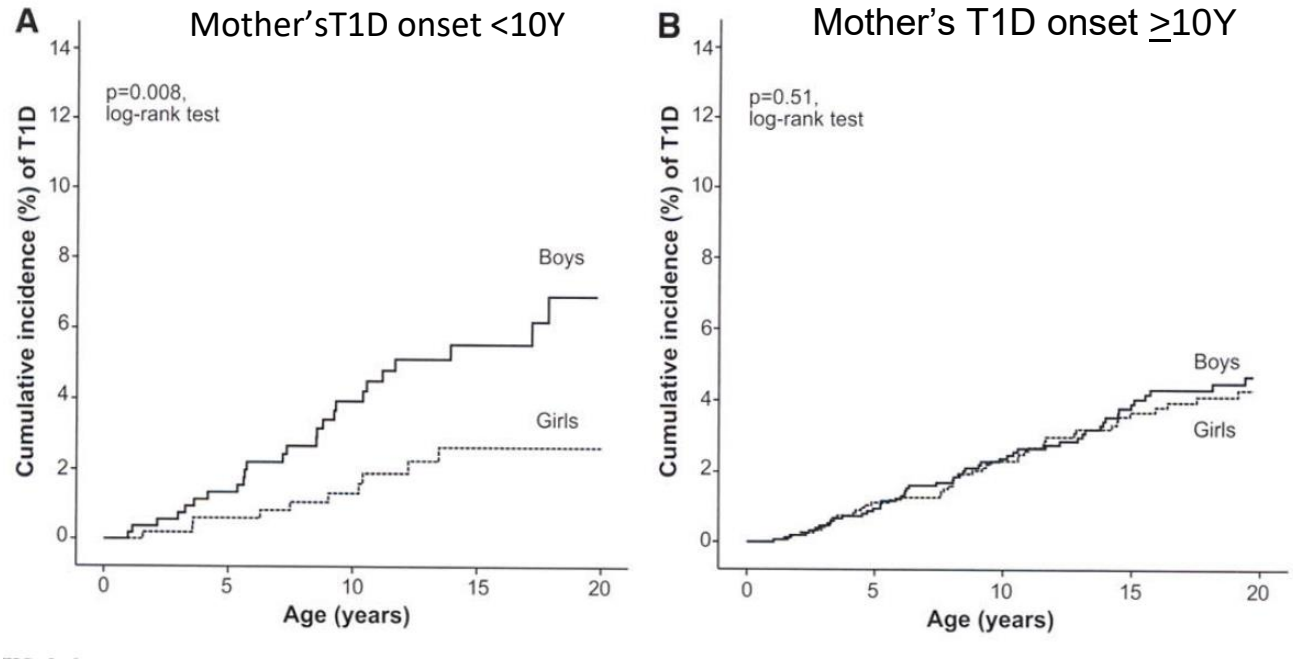


**Figure 1.** Future mothers' puberty with type 1 diabetes (T1D) affects risk to offspring by sex. Cumulative incidence of T1D in the boys and girls of the diabetic mothers with diabetes onset <10 years (*P = 0.008*, log-rank test) (A) and ≥10 years (*P = 0.75*, log-rank test) (B).

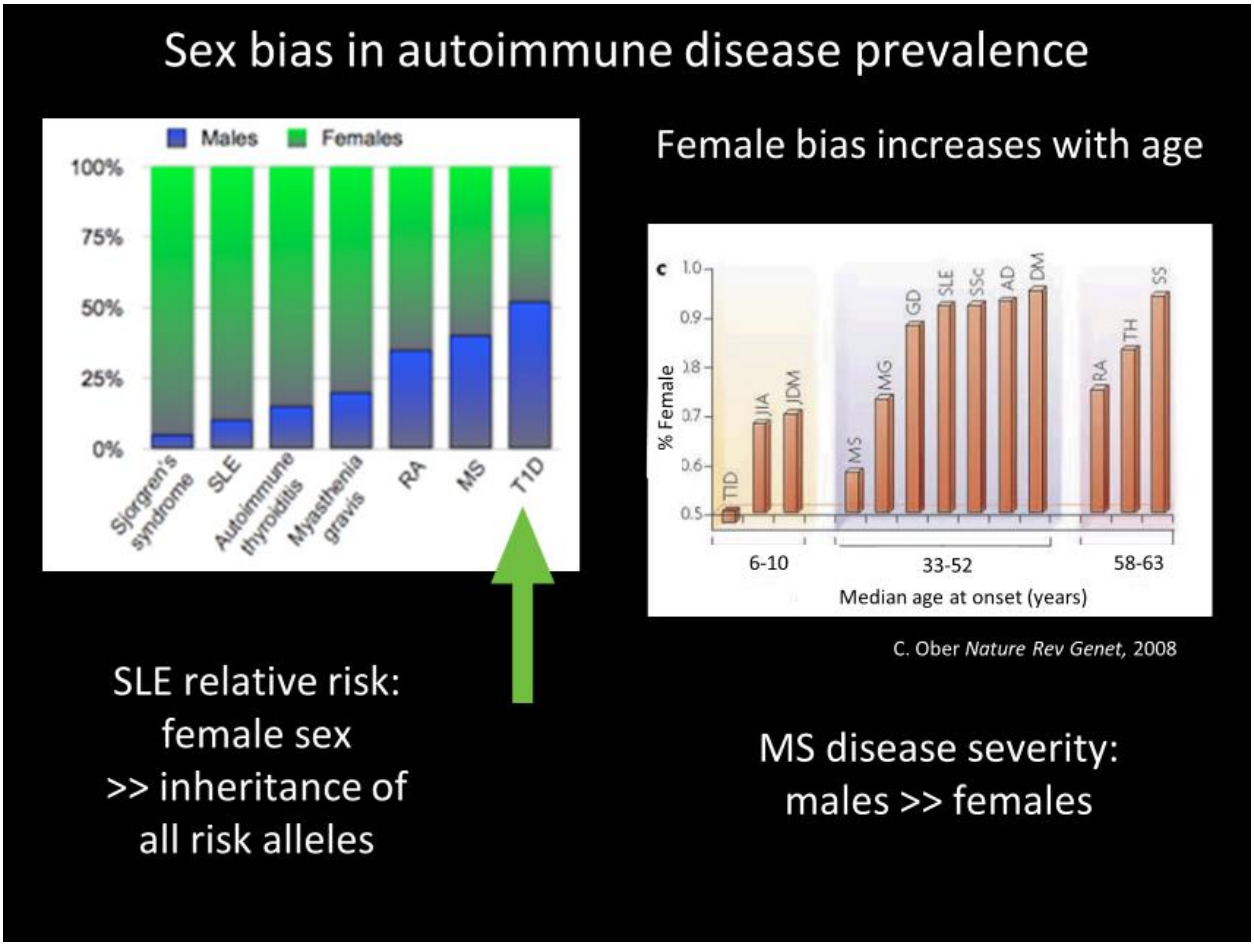


**Figure 2.** For multiple autoimmune diseases, being female confers a greater risk than any genetic factor: multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA). Sex bias in autoimmune conditions could result from direct effects of sex chromosome complement (e.g., X-linked genes), from effects of the sex hormones, or from environmental risk factors that provoke a sex-specific response.

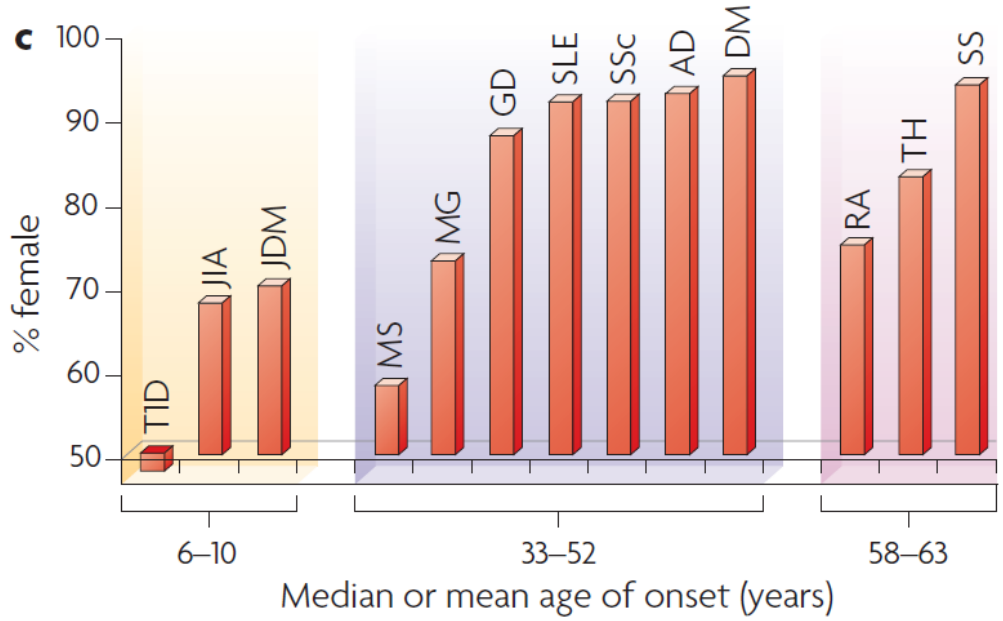


**Figure 3.** Female bias increases with age. MS disease severity: males >> females. Sex ratios (% female) by mean or median age of onset for autoimmune diseases in the U.S. and Europe. Note the female skewing at all ages, with the largest skew and number of diseases during and immediately after the reproductive years. From C. Ober, *Nature Rev Genet* (2008).

### *Discussion*

- A participant said she cannot always reproduce the same outcomes and incidence of disease in male mice and female mice, even when using the same protocols. She asked whether this was because of the microbiome. Dr. Danska replied that the microbiome may play a role, but it is most likely more complex than the microbiome alone.
- A participant asked about the role of microchimerism in explaining some sex-dependent outcomes. Dr. Danska said one of the classic experiments that started the field of immunology was microchimerism in cattle.

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## *Sex and Mouse Brain Anatomy During Health and Treatment*

***Brian Nieman, Ph.D., The Hospital for Sick Children, Toronto, ON***

Dr. Brian Nieman's research addressed long-term cognitive problems experienced by childhood cancer survivors after the curing of their primary cancers. His report focused on understanding the impact of

cranial radiation treatment for pediatric brain tumors on overall brain development. Studies show that some children undergoing cranial radiation therapy experience problems with working memory, attention, and other functions as they grow older. This ultimately can affect their performance at school and work, as well as their long-term quality of life. These effects may appear years after treatment.

His research approach focused on evaluation of brain development after radiation treatment in a mouse model and employed in vivo, whole-brain imaging approaches for quantification of anatomical change. Dr. Nieman used a computational analysis method based on registration of all images in a data set to generate an unbiased consensus average and then identify homologous features in each individual and the average. Calculation of the volume changes needed to achieve homology were then used to identify volumetric differences in the brain across time points and treatment groups.

In the studies conducted by Dr. Nieman's group, mice were irradiated 16 days after birth. They were then imaged once prior to irradiation, as well as four times post-irradiation until 14 weeks of age. When compared with controls, irradiated mice showed changes in brain volume of more than 10% in certain regions, particularly affecting the white matter. There did not seem to be a significant difference between males and females in radiation-induced change over most of the brain. However, small sex differences were seen in the volumes of the fornix and the mammillary bodies, with males showing a slightly higher rate of growth after irradiation than females.

After characterization of the developmental response to cranial radiation, Dr. Nieman's group also examined the role of inflammation in mediating radiation response. They studied the role that recruitment of peripheral monocytes in the brain could play in long-term development after radiation by treatment of mice with a knockout of the gene for chemokine (C-C motif) ligand 2 (CCL2 or MCP1). Past research has shown that knockout of the receptor for CCL2 results in improved behavioral outcomes in radiation-treated male mice.

Imaging studies showed significant volume recovery as a result of the knockout of *Ccl2* after radiation treatment in males, indicating that inflammation was mediated at least in part by *Ccl2*. However, in females, there was limited or no benefit of *Ccl2* knockout to brain development after radiation. In other words, the recruitment of peripheral immune cells via *Ccl2* seems to have an important role in males but no benefit or possibly an impairment in volume recovery in females. These results highlight an important difference between the sexes in mechanisms of altered brain development after cranial radiation, despite similar overall changes in brain development in wild-type mice.

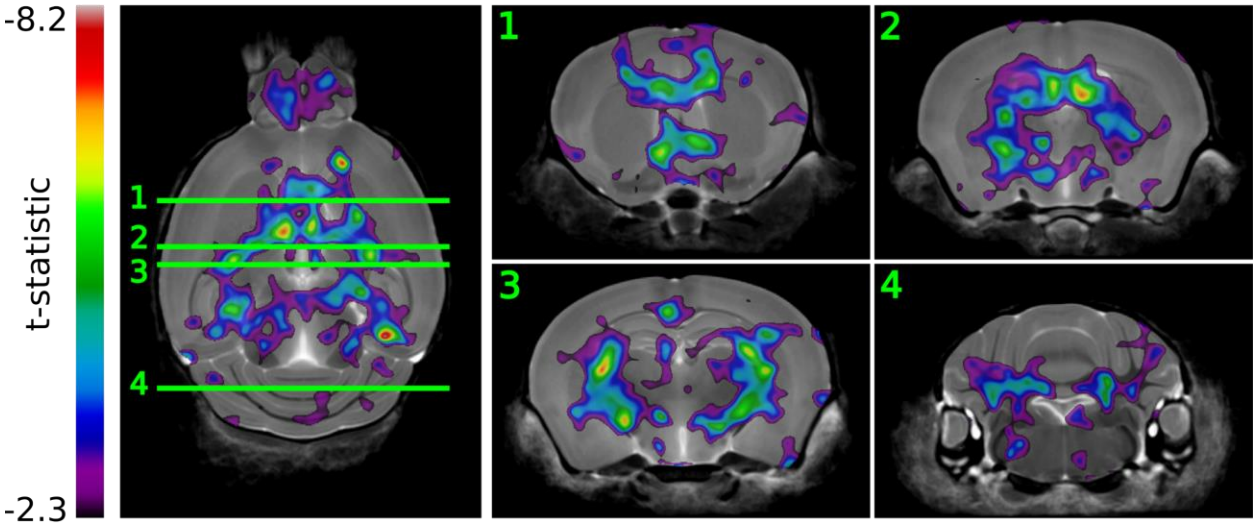


**Figure 1.** Mapping of the impact of cranial radiation over the whole brain is shown. A color overlay on top of an average MR image shows regions of significant volume decrease as a t-statistic. Radiation (7Gy) was delivered at postnatal day 16. The brain phenotype is depicted at 6 weeks of age.

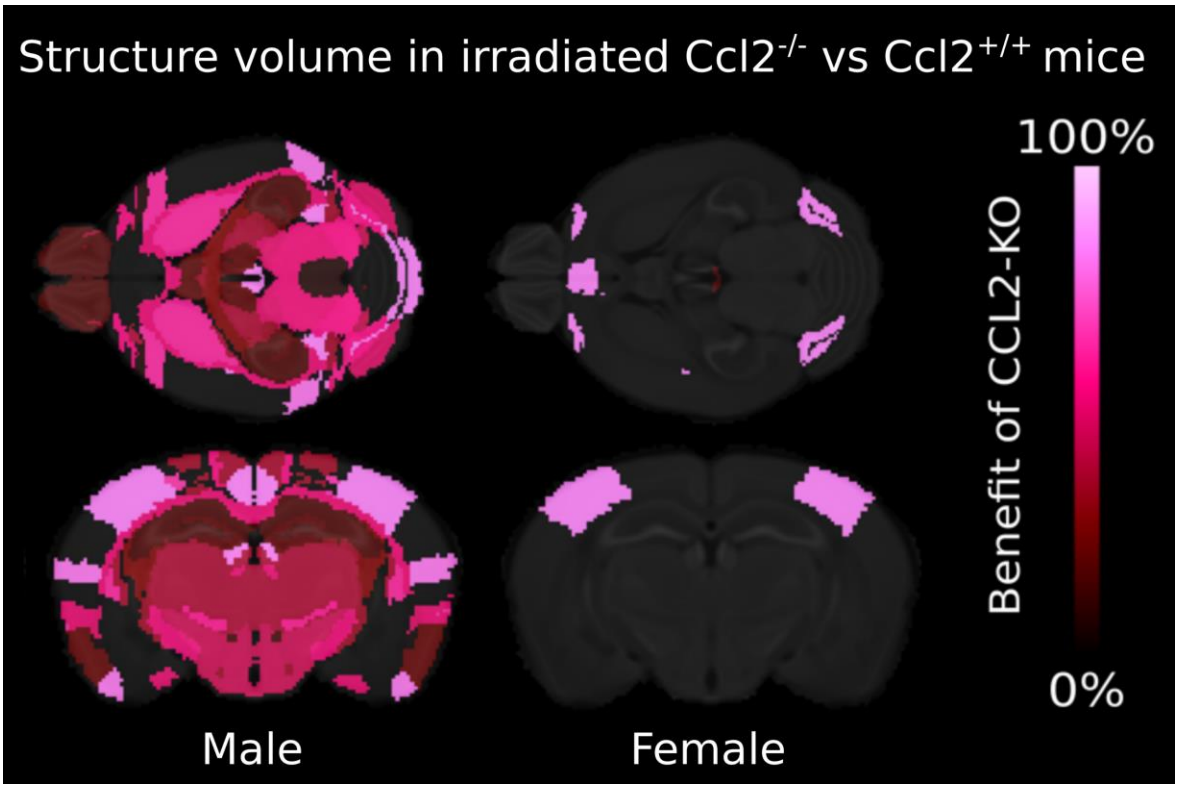


**Figure 2**. Differential impact of sex in *Ccl2* knockout mice after cranial radiation is shown. The impact of genotype on the radiation-induced volume loss (as a percentage of loss in wild-type mice, P98) is mapped across all structures in the brain for males (at left) and females (at right). Partial but widespread benefit was observed in male, but not female, mice.

## *Highlights*

- Cranial irradiation in early childhood–equivalent stages results in lasting cognitive impairments associated with developmental changes in the brain. In a mouse model, smaller volumes across much of the brain are observed in adulthood after irradiation at juvenile ages.
- Although radiation-induced volume changes in male versus female mice show only limited sex dependence, involvement of the peripheral immune system via *Ccl2* is strongly sex-dependent. This underscores that even when a similar outcome is observed between the sexes, there may be differences in pathogenic processes between males and females that have important implications for treatment.

## *Discussion*

- A participant asked whether radiation caused monocytes to massively invade the entire brain or if there was some chemical substance diffused throughout the brain to create a broad damaging/protective effect. Dr. Nieman replied that in the context of these mice, activation of microglia and recruitment of monocytes have been demonstrated. Dr. Nieman and his colleagues are conducting further studies to determine whether this occurs evenly throughout the entire brain. They also are conducting histological studies to better understand whether microglia and astrocyte activation may be different between the two sexes.
- A participant said that for some cancers, the standard central nervous system (CNS)–directed therapy is intrathecal methotrexate. She asked whether there was an opportunity to conduct a study that infuses an antiproliferative agent over a 2-year period. Dr. Nieman replied that they were studying both mouse models and humans undergoing chemotherapy treatment, including systemic and intrathecal chemotherapy. Although they had not yet conducted mouse experiments with intrathecal treatment, the systemic treatment revealed significant changes in brain development (dependent on the agent), and there appeared to be particularly strong sex effects in chemotherapy-induced changes in heart function.

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## *Estrogen Contributes to Sex Differences in M2 Polarization During Asthma*

***Aleksander Keselman, Ph.D., Johns Hopkins University, Baltimore, MD***

Dr. Aleksander Keselman stated that asthma is a chronic inflammatory obstruction of the airways that exhibits significant sex differences. The condition usually affects boys during childhood, but during puberty, the trend reverses, with females more affected in terms of incidence and severity. This could indicate a role played by sex hormones in disease progression of allergic asthma.

In asthma, cytokine secretion occurs after an allergen insult. These cytokines bind onto receptors of the alveolar macrophages, which causes the polarization of these macrophages into an M2 phenotype. This causes the secretion of chemokines and M2 proteins, which promote tissue remodeling, recruiting of inflammatory cells, enhancing of $T_H2$ responses, and fibrosis. The accumulation of macrophages in humans has been found to correlate significantly with asthma severity. Adult macrophages are therefore emerging as important mediators of allergic inflammation in the lungs, although it is not completely understood how these cells respond to sex hormones. Previous studies of bone marrow–derived macrophages and spleen macrophages suggest that sex hormones, such as estrogen, may promote M2 polarization.

To determine whether there were sex differences in macrophage polarization in the mouse model, Dr. Keselman used the ovalbumin (OVA) model for acute allergic lung inflammation. Alveolar macrophages are initiators of allergic lung inflammation. This model initiates a robust $T_H2$ inflammation, resulting in mucous production in the airways, as well as eosinophilic infiltration into the bronchoalveolar lavage.

Using flow cytometry, Dr. Keselman determined the cell expression of a variety of polarizing factors. He found that allergic female mice exhibit enhanced M2 polarization in vivo compared with male mice. Dr. Keselman then investigated whether these differences could be caused by receptor expression. He found that alveolar macrophages from female mice exhibited a higher expression of interleukin-4 receptor alpha (IL4R$\alpha$) and estrogen receptor alpha (ER$\alpha$).

In a separate in vitro experiment, Dr. Keselman used bone marrow–derived macrophages from male and female mice. He found that IL4-stimulated macrophages from female mice exhibited enhanced expression of M2 genes compared with male macrophages. This showed that some female genes are regulated in a sex-specific manner after IL4 stimulation. He also found signaling through ER$\alpha$ in macrophages with enhanced M2 gene expression.

To test the role of estrogen, Dr. Keselman used an ovariectomy model. The mice were ovariectomized at 3 weeks of age and then sensitized with OVA. Next, they were implanted with either a placebo or an estrogen-secreting pellet. The mice implanted with a placebo failed to undergo macrophage polarization to an M2 phenotype. However, if estrogen was reintroduced, it rescued M2 polarization. This suggests that estrogen is important for M2 responses in vivo.

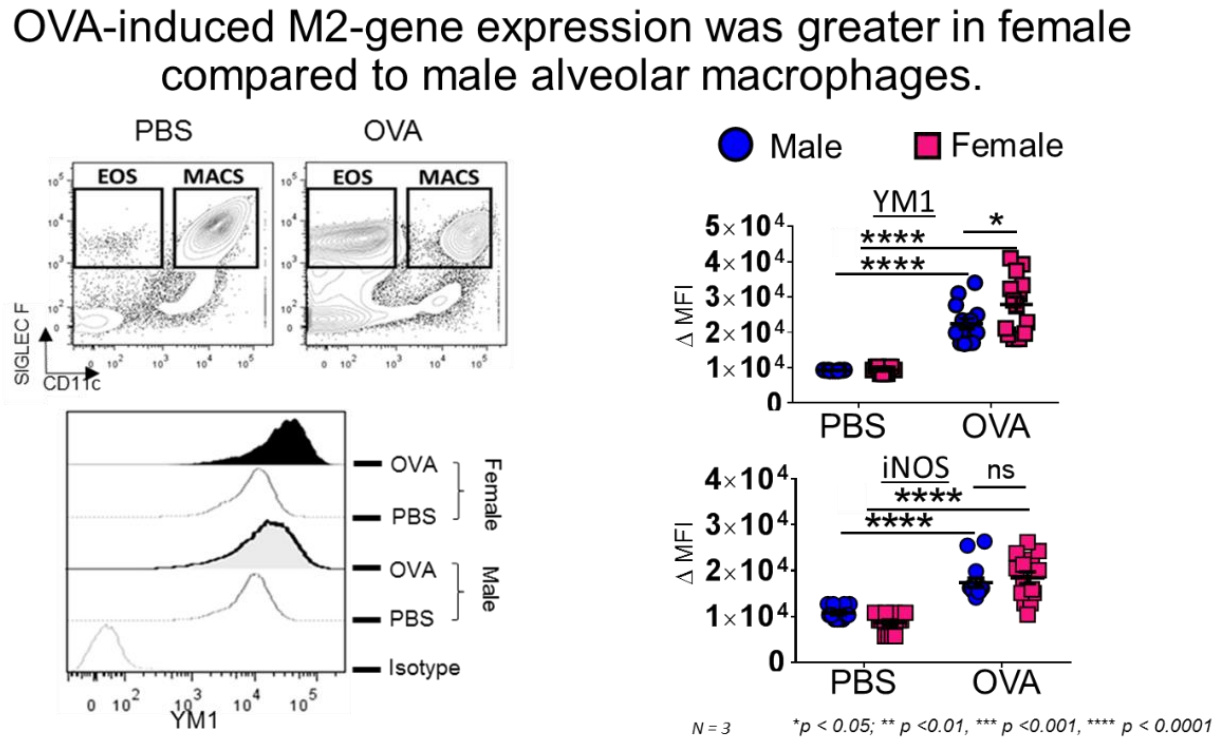


**Figure 1.** OVA-induced M2 gene expression was greater in female, compared with male alveolar macrophages.

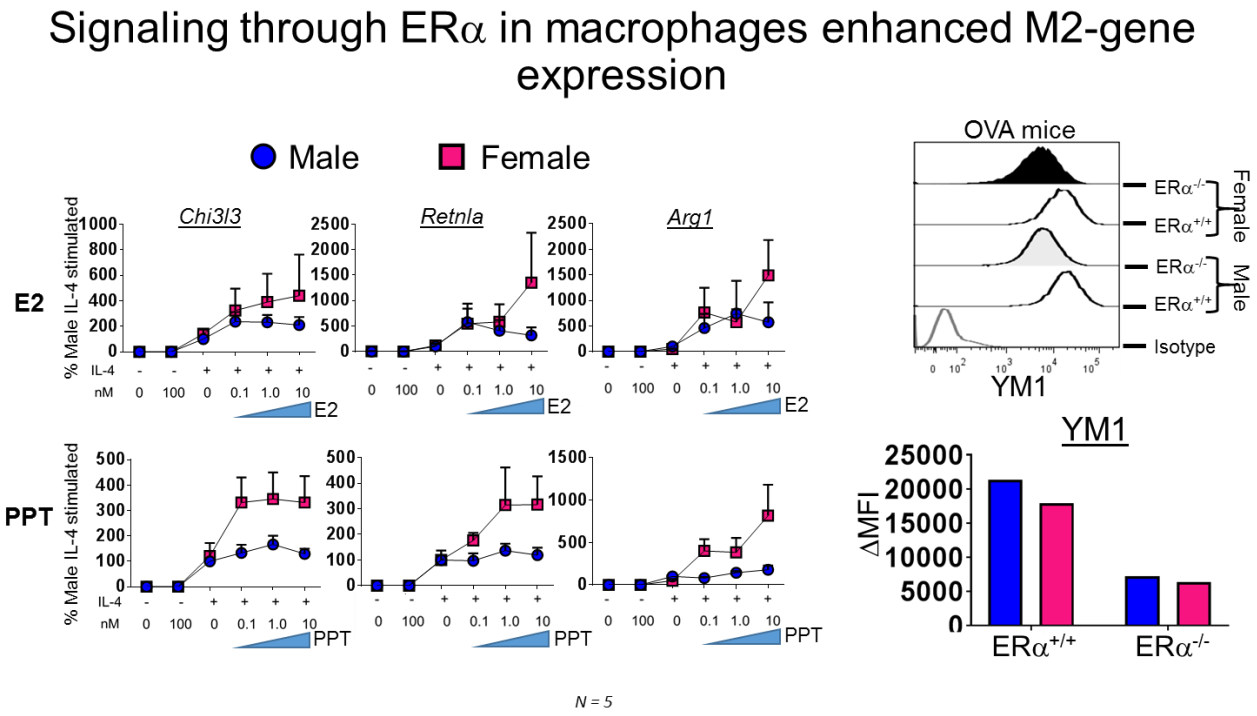


**Figure 2.** Signaling through ERα in macrophages-enhanced M2 gene expression.

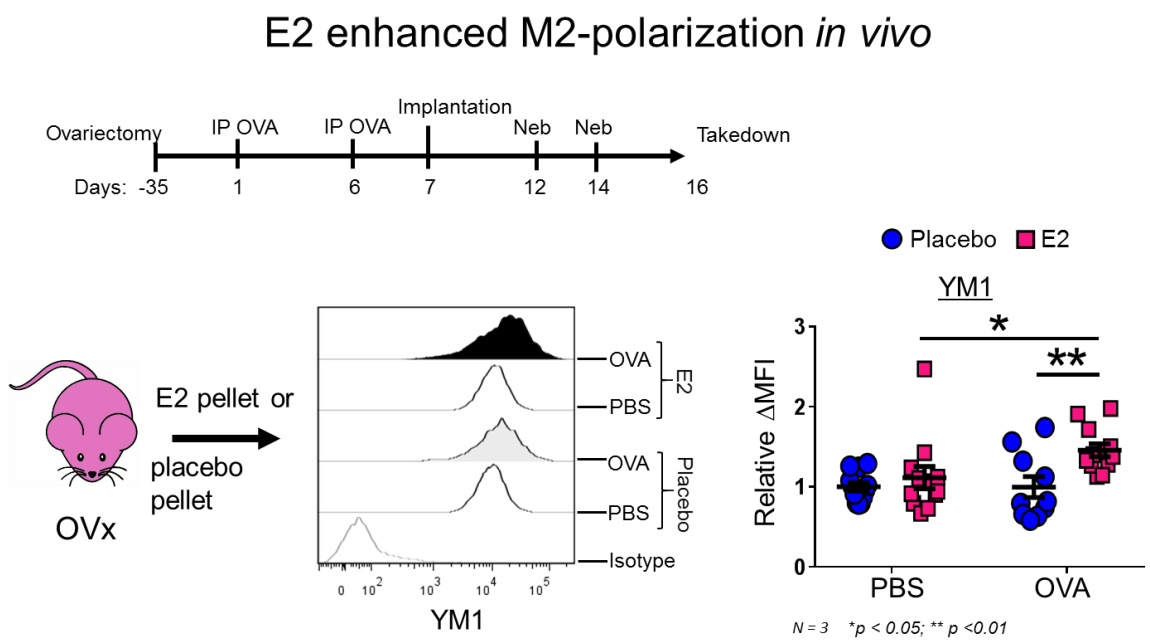


**Figure 3.** E2-enhanced M2 polarization in vivo.

## *Discussion*

- A participant asked Dr. Keselman whether he had thought about the female cycle in the context of this study, seeing as estrogen levels fluctuate. Dr. Keselman replied that the estrous cycle in the mouse is fairly short. If one has a protocol that spans weeks, mice will cycle through it. The oophorectomy model also addresses that issue, which is the reason it was used in the study.
- Another participant asked Dr. Keselman about shutting down the capacity for an M2-like polarization in response to the allergen: When one does this, does one sensitize toward M1, or does one silence macrophage activation altogether? Dr. Keselman said this depends on the cytokine environment. For example, competition between interferon gamma and $T_H1$ cytokine with $T_H2$, together with the blocking of M2 polarization, results in movement toward M1. He added that there is a great deal of flexibility with mouse models, which can be a problem. One can get different results depending on which strains/allergens are used.

## *Highlights*

- Female mice exhibit more M2-polarized alveolar macrophages after challenge with allergen than male mice.
- Macrophages from female mice express higher levels of canonical mouse M2 genes after polarization with IL4 than do macrophages from male mice.
- Ligation of ERα enhances IL4-induced M2 gene expression in macrophages from female mice, and $LysM^{CRE}ER\alpha^{fl/fl}$ mice fail to exhibit M2 polarization in the lungs after challenge with allergen.
- Ovariectomized mice fail to exhibit M2-polarized macrophages after allergen challenge, but estrogen in vivo rescues this response.

- Macrophages from female mice exhibit increased acetylation of H3 at M2 gene promoters after stimulation with IL4 than macrophages from male mice.

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## SESSION 4: POSTER SESSION

***Rajeev K. Agarwal, Ph.D., ORWH, and Paul Barrett, Ph.D., OSC (Moderators)***

Various researchers exhibited posters in the atrium of the conference center. They presented their data and analyses and were available to answer questions about their research.

## SESSION 5: SEX DIFFERENCES IN GENE EXPRESSION

***Rajeev K. Agarwal, Ph.D., ORWH (Moderator)***

### *UTI Complexity Results from Diversity at the Bacterial–Host Interface*

***Scott Hultgren, Ph.D., Washington University School of Medicine, St. Louis, MO***
***Presented by David A. Hunstad, M.D., Washington University School of Medicine, St. Louis, MO***

This presentation focused on translating basic science into novel therapeutics for urinary tract infections (UTIs), which can become chronic, recurrent bladder infections. There are approximately 10–15 million cases of UTIs every year, at a cost of over $2.5 billion. In addition, catheter-associated UTIs add nearly $1 billion in health care costs annually.

Both community- and hospital-onset infections are caused by uropathogenic *Escherichia coli* (UPEC). *E. coli* bacteria can be difficult to eliminate because they can enter the bladder's cells and replicate extensively inside, creating bacterial communities. Because these communities hide inside living cells, it is difficult for the body's immune system to detect and eliminate them. Also, bacteria are becoming more difficult to treat because of multi-drug resistance.

One hypothesis is that UTIs are caused by *E. coli* harbored in the host's microbiome. Researchers have determined that UPECs are not genetically homogeneous. The hypothesis is that both the host and the bacteria have a combination of factors that can cause a UTI when there is synergy between them. These interactions can change based on genetics, environmental factors, a history of previous UTIs, and sex differences. As a result, a UPEC strain that may cause a UTI in one woman may not cause an infection in another.

One of the most important host determinants is a history of UTIs. Dr. Hultgren's lab found that recurrent UTIs in mice anatomically change the surface of the mouse bladder. After the UTI is treated, the epithelial surface does not return to its previous state. Instead, the anatomical change in the mice is permanent, and the bladder tissue is considered "sensitized." Sensitized tissue not only looks anatomically different but behaves differently. When these mice are re-exposed to bacteria, they are more likely to become infected at a smaller inoculant dose or become infected when inoculated with bacteria that would not cause a UTI in naive mice.

One strategy being developed to treat UTI infections is treatment without antibiotics. UPEC and other bacteria use a variety of surface adhesive fibers, called pili, as well as molecules, to stick to epithelial cells in the bladder, which is the instigating event for a UTI. Pilicides, a class of molecules used in Dr. Hultgren's lab, have been found to kill the molecular machine that assembles the pili, completely shutting off pili production. This keeps the bacteria from sticking to cells and causing an infection. Yet pilicides do not kill the bacteria, which means they do not impose the natural selective pressure for the development of antibiotic resistance.

This approach uses an antivirulance compound rather than an antibiotic. Dr. Hultgren and colleagues, along with a company called Fimbrion, are developing mannosides that can block bacterial binding to the bladder. Studies in mice have shown that use of these mannosides can significantly lower the bladder bacterial burden. Studies also have shown that mannosides given orally can deplete type 1 UPECs in the gut without depleting the rest of the microbiota, while simultaneously treating UTIs.

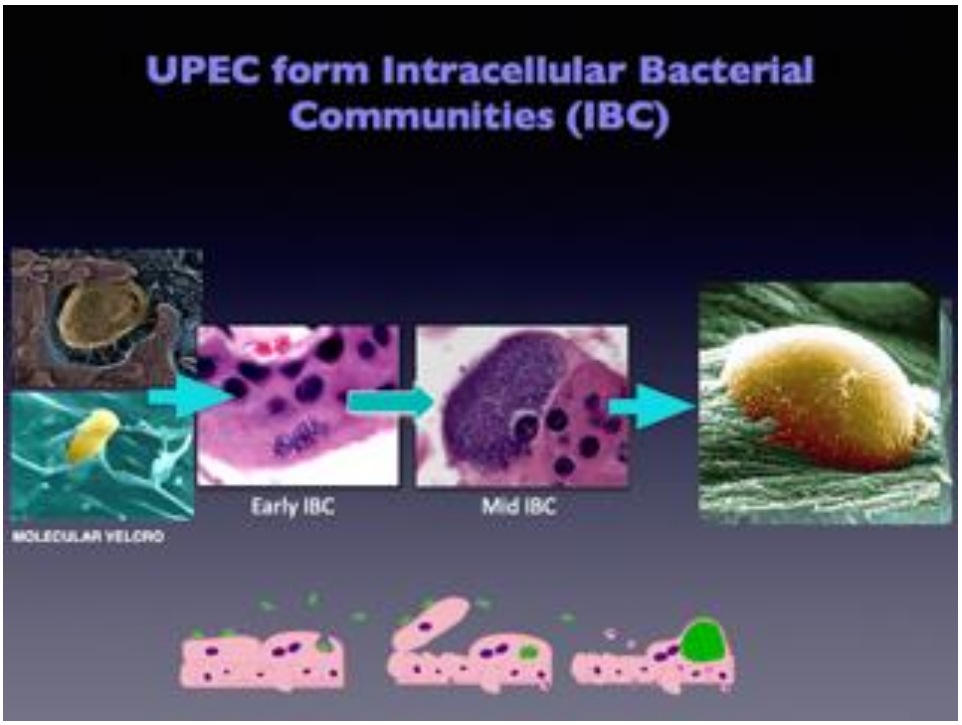


**Figure 1.** UPECs form intracellular bacterial communities (IBCs).

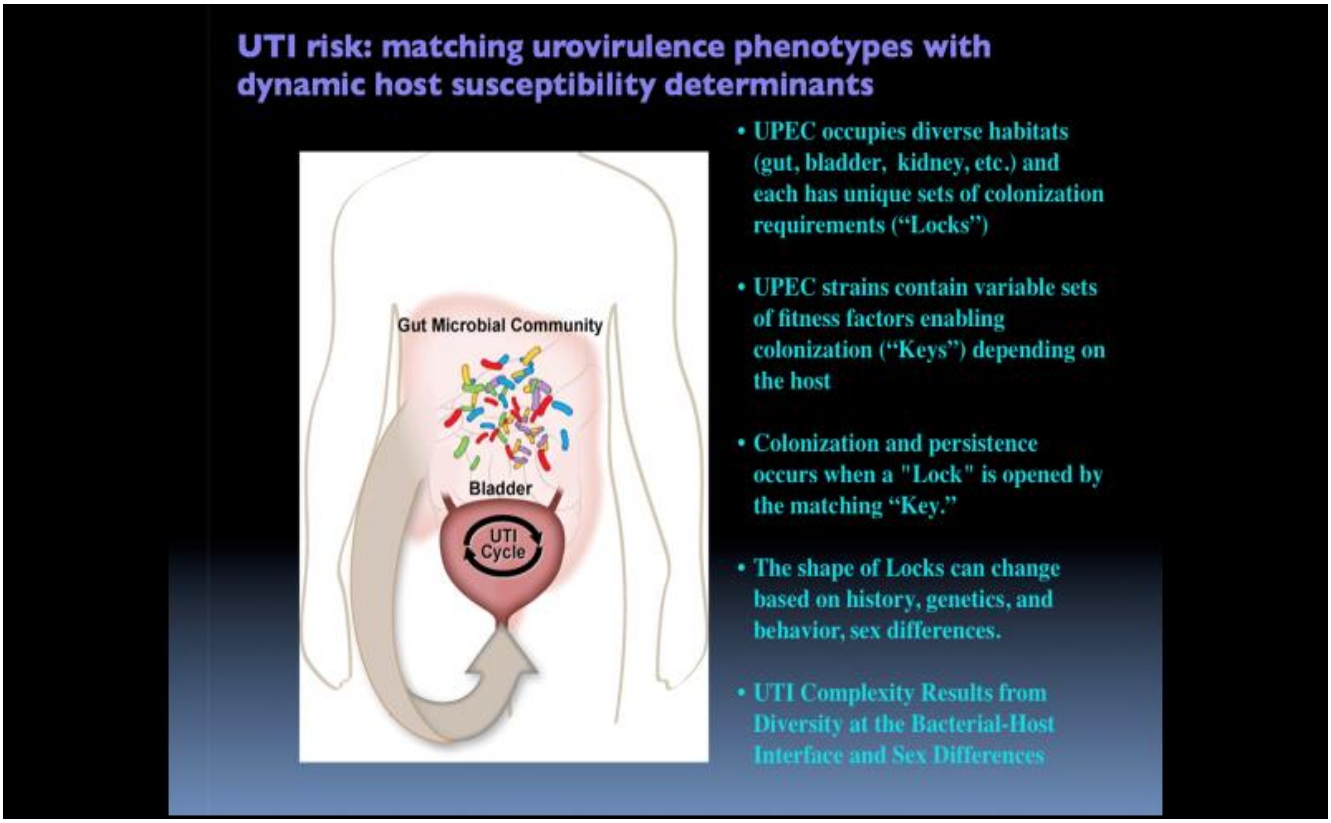


**Figure 2.** UTI risk: matching urovirulence phenotypes with dynamic host susceptibility determinants.

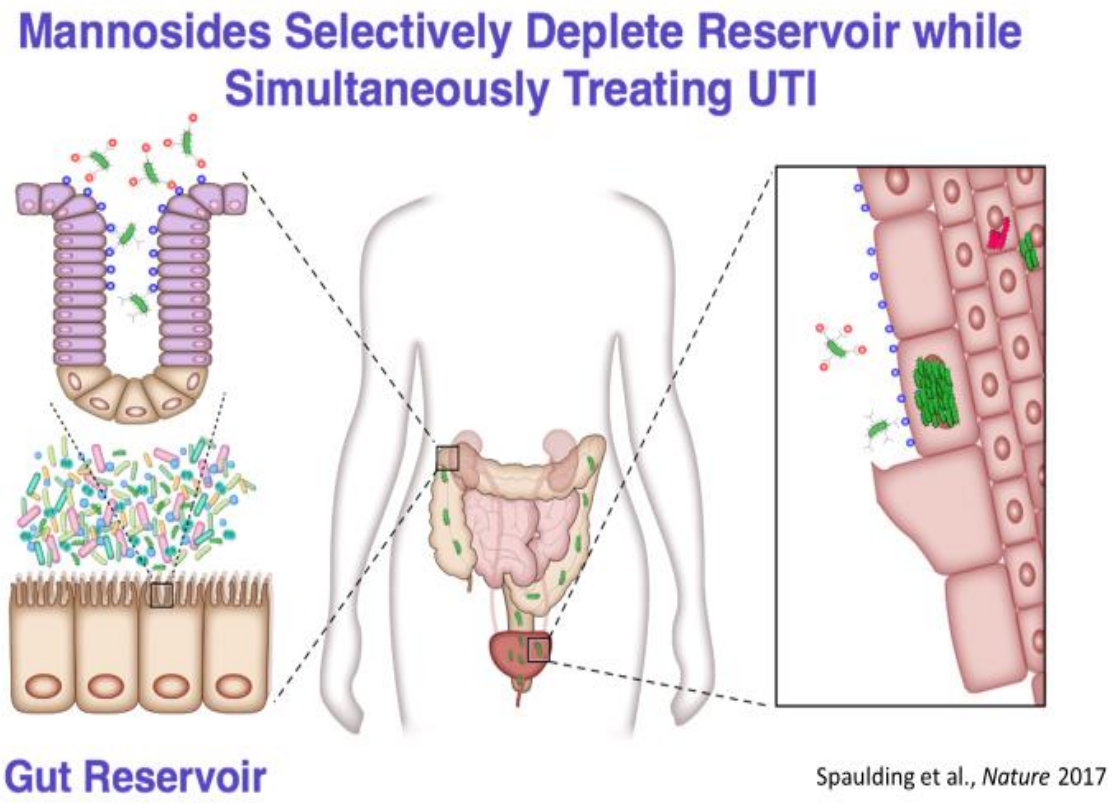


**Figure 3.** Mannosides selectively deplete reservoir while simultaneously treating UTI.

## *Highlights*

- Studies have suggested a "lock and key" model of UTI pathogenesis, in which the ability of a particular *E. coli* isolate to cause UTI in a particular human is dependent on the "match" between a bacterial strain and the host it is inhabiting.
- UTIs are most often caused by one of a diverse collection of uropathogenic *E. coli* (UPEC). Overall, UPECs share as little as 60% of their genes. Research on diverse UPECs suggests that the expression of core genes correlates with infection.
- Differences in host factors—including differing genetics, sex, age, and history of previous infections—can have a significant impact on the ability of the same strain and different strains to cause UTIs.
- Understanding the pathogenic mechanisms that UPECs use to cause UTIs, including critical adhesive interactions, has led to the development of anti-virulence therapeutics, which can efficiently remove UPECs from the urinary tract and from their reservoir niche in the gut without causing deleterious effects on the host's beneficial microbiota community.

### *Discussion*

- A participant asked whether Dr. Hultgren's team was looking at the transcriptome or metabolome of different bacteria. Dr. Hunstad said his team was looking at the transcriptome of the bacteria in the gut versus the bladder and examining the transcriptome of bacteria in the lumen of the bladder versus those growing inside epithelial cells. This was taking place in collaboration with the Broad Institute.
- A participant asked whether the healthy gut microbiome contains uropathogenic *E. coli*. Dr. Hunstad replied that *E. coli* is a minor component of the microbiome compared with anaerobes and other bacteria. There are some *E. coli* strains that may have virulence determinants that would allow them, under the right circumstances, to cause a UTI in some, but not all, women. This is because of the complicated relationships of the virulence factors expressed by the bacteria and the permissive factors that are features of the host.

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## *Sex Differences in Pathogenesis of Urinary Tract Infection*


***David A. Hunstad, M.D., Washington University School of Medicine, St. Louis, MO***


Dr. David Hunstad's presentation explored potential sex differences in UTIs. While some people think UTIs affect only women, about 3–5% of girls and about 1% of boys also have had a UTI before puberty. This ratio changes significantly in adulthood, with 50% of young and middle-aged women experiencing at least one UTI and nearly no males in this group experiencing an infection. However, catheterized males or males with spinal cord injuries also can suffer from UTIs.

The hypothesized reason for this sex discrepancy in UTIs has traditionally been attributed to anatomical factors—a shorter distance from the anus to the urethra in females, as well as a shorter urethral length. Dr. Hunstad's lab used the murine model to determine whether there are factors beyond anatomy that favor UTIs in females.

One of the barriers for studying UTIs in male mice is that female mice can be inoculated via catheter whereas male mice cannot. To circumvent this issue, Dr. Hunstad inoculated C3H mice by creating 2–3-millimeter incisions in the abdominal walls to reach the bladders and then injected them with *E. coli* bacteria using needles. This model showed no apparent peritonitis and had a 99.3% survival rate.

When both female and male mice were inoculated using this procedure, the bacterial loads in the bladder were slightly lower for females 6 hours after inoculation but were nearly the same at 24 hours. After 2 weeks, however, nearly 100% of the males had developed bacterial loads typically seen in chronic cystitis, compared with approximately 25% of females. Also, when compared with female bacterial loads, male bacterial loads were significantly higher. In addition, when compared with female mice, males exhibited far more extensive kidney disease. (See Figure 1, left panel.) More than 90% of males exhibited renal abscesses (see Figure 1, right panel), which are rare in females. This led to further research to determine the impact of hormones in mice with chronic cystitis.

A study showed that ovariectomized female mice showed no real differences in bladder or kidney loads when compared with sham females. However, castrated males showed a much lower bacterial load 2 weeks post-infection when compared with sham males. In other words, castration prior to infection was found to be protective against severe UTI outcomes.

Dr. Hunstad's lab developed a mouse study that showed that dihydrotestosterone (DHT), a strong and specific androgen receptor agonist, promotes severe UTI outcomes in castrated males compared with castrated males who are given a placebo. (See Figure 2A.) In fact, DHT given to females also was found to induce susceptibility to severe UTIs. (See Figure 2B.) These and other data presented demonstrate that androgen receptor activation potentiates UTI susceptibility and severity in both host sexes. As a corollary, the anatomical aspects of the urinary tract were shown to be protective in males.

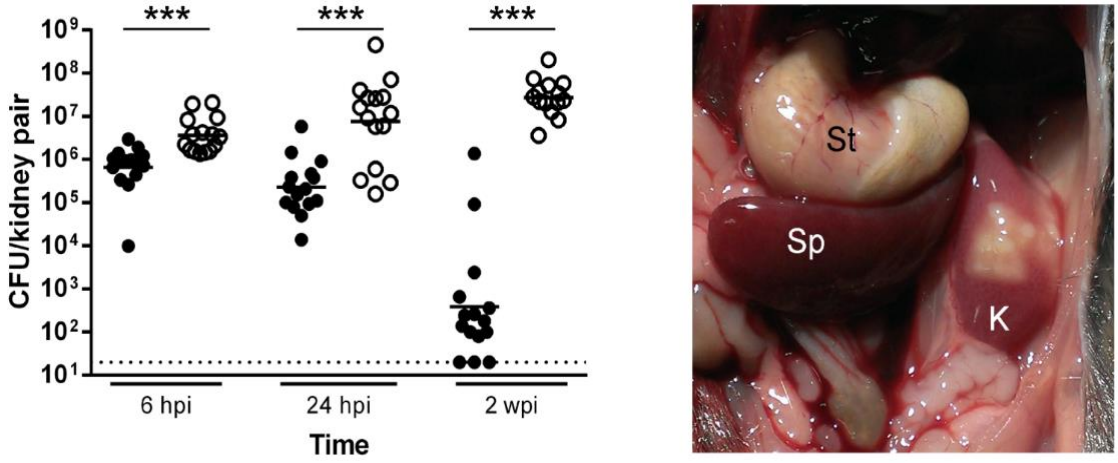


**Figure 1.** Left panel: Bacterial loads (colony-forming units [CFU]) of uropathogenic *E. coli* recovered from the kidneys of female (filled circles) or male (open circles) C3H/HeN mice at the indicated time points. ***, $p<0.001$ by Mann-Whitney U test. Right panel: Example of a grossly evident abscess on the left kidney (K) of an infected male mouse 2 wpi (St, stomach; Sp, spleen). Adapted from *J Am Soc Nephrol* 2016; 27: 1625–1634.

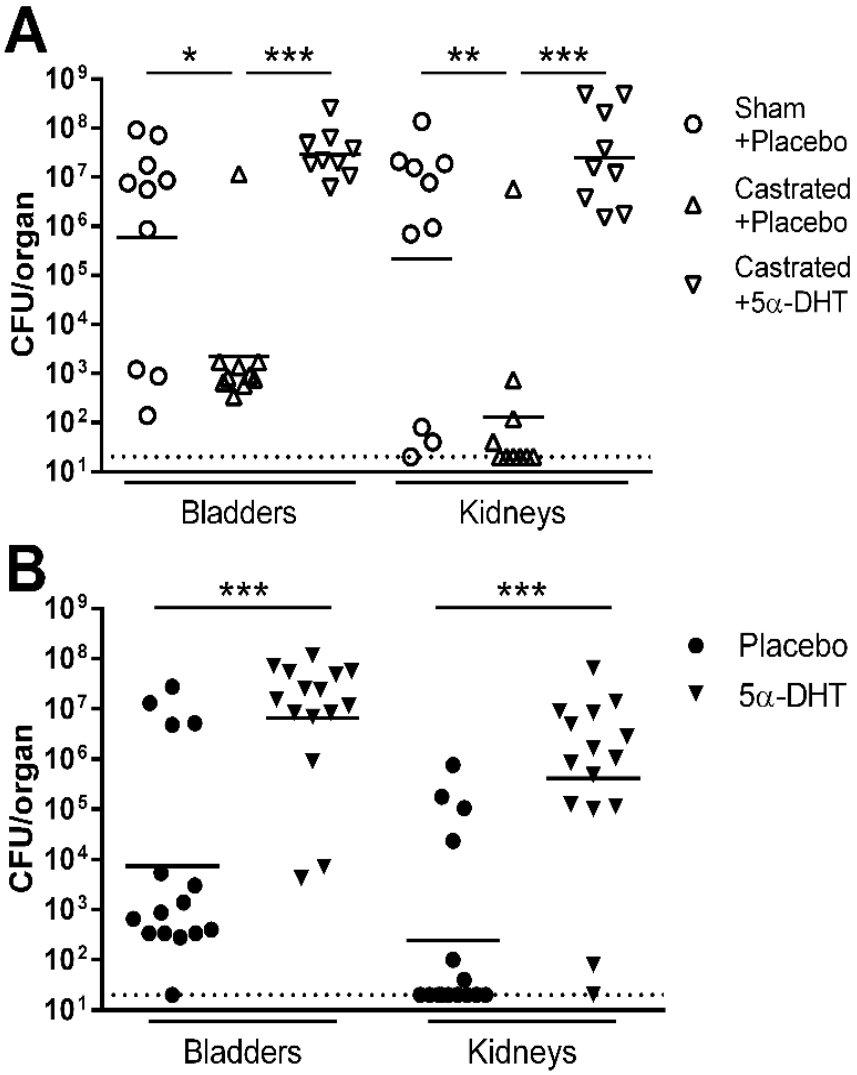


**Figure 2.** Bacterial loads 2 weeks post-infection in bladders and kidneys of (A) male C3H/HeN mice, either sham-operated or castrated and then given DHT or placebo, or (B) female C3H/HeN mice given DHT or placebo.

## *Discussion*

- A participant asked whether the androgen was acting in the bladder or the kidney. Dr. Hunstad said he believed it acted in both the bladder and the kidney. He and his team were exploring ways to physically separate those effects.
- Another participant asked about receptor expression in the two organs. Dr. Hunstad said the androgen receptor is expressed widely. Renal tubular epithelium, bladder epithelium, and immune cells that are present and recruited to both organs are expected to express the androgen receptor. He and his team were working on developing these data for the C3H model.

- A third individual asked whether chronic UTI or the persistence of bacteria in the bladder epithelium are related to the symptom complex called “interstitial cystitis” or “chronic bladder pain.” Dr. Hunstad said this is not yet known.
- Lastly, a participant asked whether there was engagement of a membrane-associated androgen receptor. Dr. Hunstad said he and his team would pursue that question as they continue to drill down on mechanisms.

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- Olson, P. D., McLellan, L. K., Hreha, T. N., Liu, A., Briden, K. E., Hruska, K. A., et al. (*In revision*). Androgen exposure potentiates formation of intratubular communities and renal abscesses by *Escherichia coli*.


## *X, Drugs, Rock, an’ Roles…*


***Lauren A. Weiss, Ph.D.; University of California, San Francisco; San Francisco, CA***


Dr. Lauren Weiss developed and tested four hypotheses in autism and nine additional heritable diseases for comparison. The hypotheses focused on studying the following: (1) contribution of the X chromosome, (2) contribution of steroid-responsive genes, (3) burden of genome-wide liability, and (4) role of anthropometric sex-heterogeneous single nucleotide polymorphisms (SNPs).

For autism, a sample was studied that included approximately 8,000 males and 2,000 females. The top five combined results showed involvement of SNPs near gene EXT1 on chromosome 8, gene EXOC4 on chromosome 7, and other genes on the X chromosome. When disaggregated, the results for males showed involvement of the same EXT1 region on chromosome 8 and various loci on the X chromosome. However, for females, the top results showed SNP associations on chromosomes 2, 7, and 8. For chromosome 8, the locus was different from the chromosome 8 locus associated with autism in males (CSDM1). In other words, the top results in the female group were female-specific autism loci, showing that there was a sex difference in genetic architecture.

With respect to the genetic burden, a prevalent hypothesis called “the liability threshold model” states that there is a higher genetic risk needed for females than there is for males to obtain a diagnosis of autism. This is consistent with the lower prevalence seen in females.

Polygenic risk scores were calculated from male data to determine genome-wide risk burden for common polymorphism risk. No differences were observed between female and male case or control polygenic risk distributions. There was no evidence that higher heritability was associated with the lower-prevalence sex in any of the additional nine diseases. These data do not support the liability threshold model with respect to polygenic risk.

A different analysis examined the contribution, or potential enrichment, of androgen-responsive or estrogen-responsive genes. There was no signal in the gene sets in autism. However, there was a signal in three of the other nine common diseases studied (i.e., rheumatoid arthritis, coronary artery disease, and type 1 diabetes), as well as in almost all of the anthropometric traits studied (e.g., height, weight, body mass index, and waist–hip ratio). This finding shows that sex hormones have an impact on many complex traits, but no role of sex hormones was detected for autism by this approach.

There also was evidence for enrichment in a group of SNPs showing sex heterogeneity of effects on anthropometric traits. This was observed in autism, in five of the nine other complex disorders, and in all anthropometric traits. Similar biological mechanisms may underlie many of these sex differences.

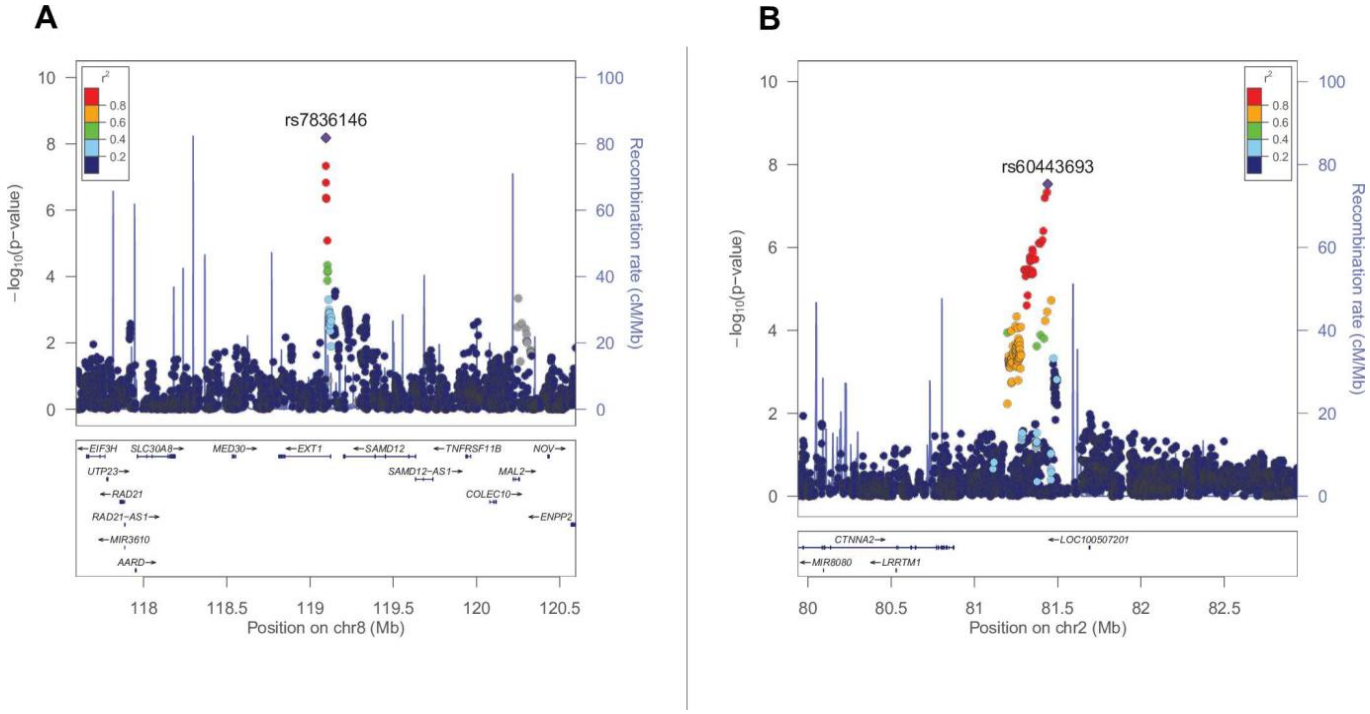


**Figure 1.** Plot of region surrounding most significant SNPs. (A) Male ASD association results surrounding rs7836146 in the region on chromosome 8: 117.6±120.5 Mbp. (B) Female ASD association results surrounding rs60443693 in the region on chromosome 2: 79.9±82.9 Mbp. Plots were generated using LocusZoom. SNP position information was based on hg19 reference version, and linkage disequilibrium (LD) and recombination rate data were based on 1,000 genomes (November 2014) in the European population. SNPs are colored based on LD correlation (r2) or colored gray if no LD information exists. The overlaid blue line corresponds to the recombination rate.

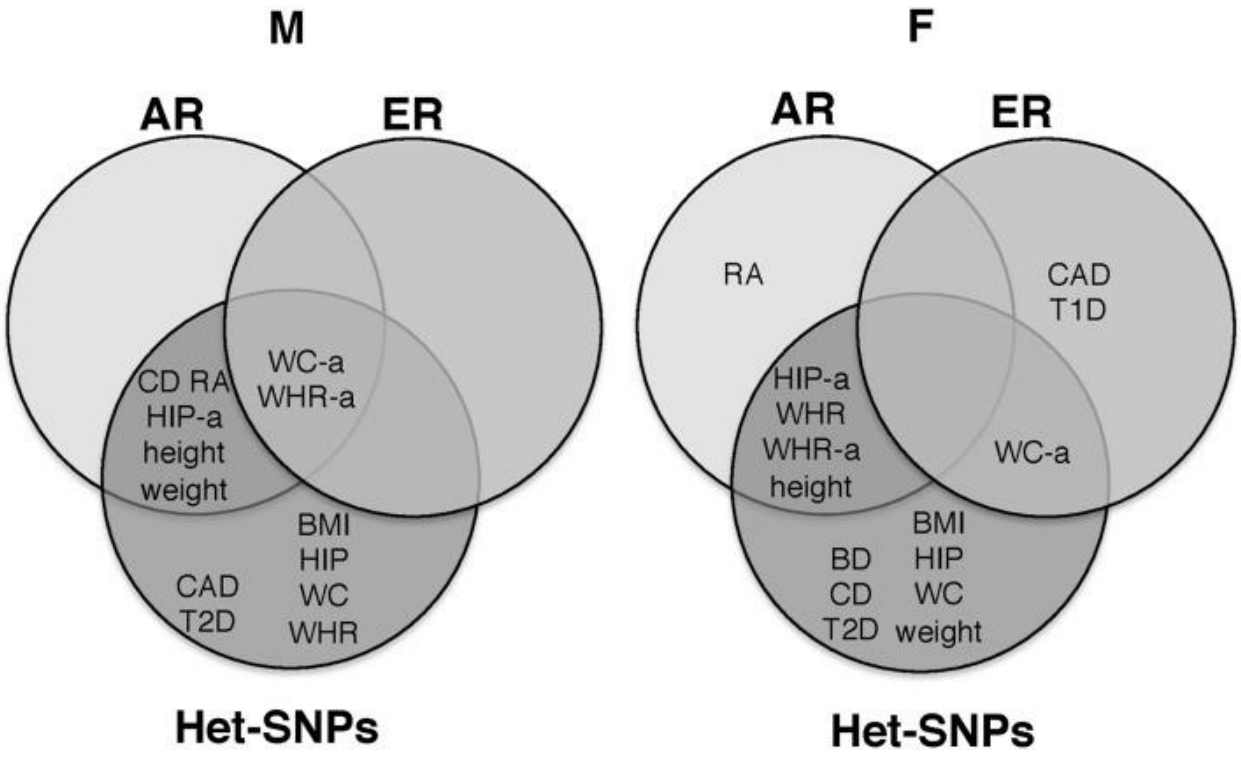


**Figure 2.** Venn diagrams of association enrichment of androgen-responsive (AR) and estrogen-responsive (ER) genes and heterogeneous SNPs (het SNPs) in WTCCC diseases and GIANT anthropometric traits. Diseases/traits with significant association enrichment in males (M) are represented in the left diagram; diseases/traits with significant association enrichment in females (F) are represented in the right diagram.

## *Discussion*

- A participant asked about the data presented regarding the X-linked SNPs in the genome-wide association study (GWAS) data for autism. She said that some of the minor allele frequencies were low. She asked whether there was any information about population stratification. Dr. Weiss said they are always concerned about stratification and explained that most of their data is family-based to eliminate stratification. Also, because they used some strategies to correct for potential stratification, it was not believed to be an artifact.
- Another participant asked whether any SNPs and anthropometric measures examined were related to inflammation. The participant said she had postulated a similar basal inflammation threshold model that, when presented with an insult, trips over to dysregulation. Dr. Weiss said her team's planned next step was to develop unbiased enrichment testing to determine what the regions detected as sex-heterogeneous in anthropometric traits have in common.

## *Highlights*

- Genetic approaches can efficiently address biological hypotheses about sex differences in human disease.
- There was some evidence for X chromosome contributions to disease, including increased sex heterogeneity and male-limited effects.

- There was also some evidence for steroid hormone contributions via gene expression to common diseases (although not autism), as well as substantial evidence for such contributions to anthropometric traits.
- The investigators did not find strong support for a common polymorphism liability threshold model to explain sex-differential prevalence in common diseases.
- There was evidence for pleiotropy between disease risk and sex-specific anthropometric trait-associated polymorphisms.

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## *Sex-Specific Genetic Architecture of the Human Transcriptome*


***Barbara Stranger, Ph.D., University of Chicago, Chicago, IL***


Dr. Barbara Stranger focused on the influence of sex in gene expression. Normal cell health, function, and development depend on the right genes being turned on at the right time and in the right place. Altered patterns of gene expression can sometimes lead to disease.

In every species, there is variation among individuals with respect to gene expression levels. Much of this has a genetic component. Studies in humans and other species have mapped the locations in the genome where the genetic effects that regulate gene expression levels lie. Some variants associated with disease also are associated with gene expression.

Many diseases display a sex bias in prevalence, clinical features, and diagnosis. Also, some traits and diseases have significant sex differences in heritability, such as triglycerides, HDL, LDL, and systolic blood pressure. These differences have traditionally been attributed to two factors: (1) different hormone levels between the sexes or (2) sex chromosome genes. Dr. Stranger postulated that autosomal genes also may play a part in these differences.

Dr. Stranger's study made use of the NIH Genotype-Tissue Expression (GTEx) program. GTEx spurred the collection of approximately 50 types of human tissues, as well as 10 brain regions from over 900 deceased donors. In addition to using the GTEx database, researchers can request biobanked tissue samples for their research. GTEx allows researchers to study correlations between genotype and tissue-specific gene expression levels. This helps them identify regions of the genome that influence whether a gene is expressed and, if it is, to what degree.

Dr. Stranger examined data for 20 tissues from more than 120 individuals. She examined SNP-by-sex interactions for each tissue. The goal was to determine whether expression quantitative trait loci

(eQTLs) behave in a sex-dependent manner. She presented results indicating that for subcutaneous adipose tissue, the STK32C gene showed opposite effects by sex. However, Dr. Stranger emphasized that when the data were aggregated, this association was lost, making it appear as if there was no effect. Researchers looking at the aggregated data would not find a sex effect. Similar results were seen for the MXRA8 gene. For the tibial nerve gene GTPBP1, there was no significant effect in males but a strong significant effect in females.

In all, approximately 200 SNP-by-sex interactions were found across tissues, although the authors were still assessing the best way to control multiple testing in this context. A conservative view would be that only several hundred genes across tissues exhibited sex differences in genetic regulation of expression. The X chromosome was not found to be enriched for interactions. Genes also were not enriched for sex-related functions. Dr. Stranger emphasized that research was ongoing. Therefore, the conclusions could change when the data are fully analyzed.

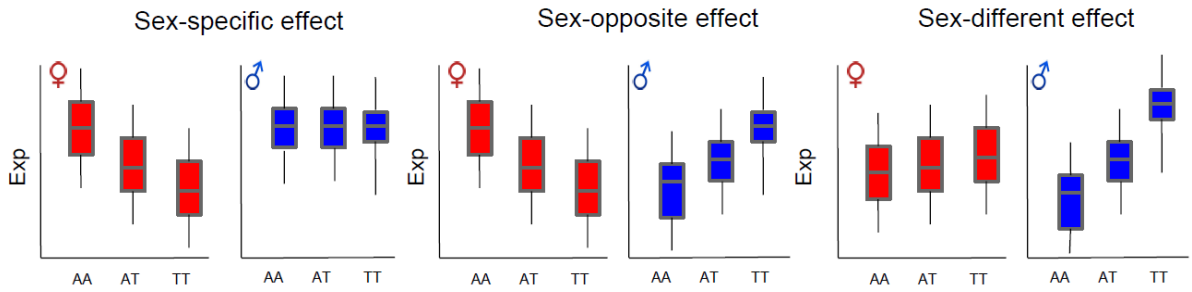


**Figure 1.** Sex differences are shown in the genetic regulation of gene expression levels. The figure illustrates hypothetical examples of (1) a sex-specific effect, in which the genotype of a particular SNP is associated with expression levels of a given gene in only one sex, (2) a sex-opposite effect, in which the genotype of a particular SNP is associated with gene expression levels of a given gene in both sexes but the allelic direction of effects is flipped between sexes, and (3) a sex-different effect, in which the genotype of a particular SNP is associated with gene expression levels of a given gene in both sexes but the magnitude of the effect differs between sexes.

## *Highlights*

- Sex differences in gene expression levels and splicing in humans are not limited to the genes located on the sex chromosomes. Sex differences in autosomal gene expression are a common feature of human tissues, although the fold change between males and females is typically quite small.
- Investigators identified sex differences in the genetic component of gene expression levels in human tissues, with some associations involving SNPs and genes previously implicated in human complex traits or diseases.
- SNP-by-sex interaction eQTLs are difficult to detect statistically for reasons related to power and inter-individual heterogeneity.
- Current eQTL studies are most likely underpowered to detect SNP-by-sex interaction eQTLs.
- SNP-by-sex interaction eQTLs appear to be highly tissue-specific, but this may be a consequence of low power.
- eQTL studies have proved useful in annotating GWAS results and uncovering the genes, mechanisms, and biology underlying human quantitative traits and disease. The researchers expected that as eQTL study sample sizes increased, SNP-by-sex interaction eQTLs would be

useful for understanding the biology and mechanisms contributing to human complex traits with sex-differentiated characteristics.

### *Discussion*

- A participant asked whether Dr. Stranger had examined the data as a matrix of SNPs by tissues, representing each cell as the magnitude of the estimated SNP-by-sex interaction. She asked whether tissues would "show clustering" in a matrix such as this. Dr. Stranger replied that she and her team had not examined the data that way. She stated that if one looks at differential expression, or splicing, there is definitely tissue clustering as well as tissue similarity in those metrics.
- A participant asked whether all donors were adults. Dr. Stranger said they were. There were some young adults, but most donors were older individuals. Most of the women were postmenopausal.

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## SESSION 6: PANEL DISCUSSION ON LESSONS LEARNED: ACCOUNTING FOR SABV

***Moderator:***
***Judy Hewitt, Ph.D., National Institute of Allergy and Infectious Diseases (NIAID)***

***Panel Members:***
***Emeran A. Mayer, M.D.; University of California, Los Angeles; Los Angeles, CA***
***Margaret M. McCarthy, Ph.D., University of Maryland School of Medicine, Baltimore, MD***
***Virginia M. Miller, Ph.D., Mayo Clinic, Rochester, MN***
***Brian Nieman, Ph.D., The Hospital for Sick Children, Toronto, ON***
***Michèle Ramsay, Ph.D., University of the Witwatersrand, Johannesburg, South Africa***

### *Panel Discussion*

Each panel member explained how and why he or she began studying sex differences. The floor was then opened for participant questions and discussion. Dr. Judy Hewitt, the moderator, asked the panelists how they would encourage researchers to include both sexes in their studies and how they could be encouraged to analyze their data in more depth.

Dr. Virginia Miller said she would tell investigators that they were missing opportunities, because they could modify their approaches and become eligible to apply for grants that support SABV research. Some investigators' research blossomed because they applied for specific requests for applications (RFAs) and subsequent R01 grants.

Dr. Margaret McCarthy noted that many investigators have concerns about the estrous cycle. They incorrectly believe that using female mice would require doubling the number of samples or even greater increases in sample size. However, three meta-analyses showed that the variability introduced by the estrous cycle can be less than that introduced by housing conditions. There is also research indicating that one does not have to double the sample size to study both sexes. For example, if the normal sample size is eight and the Cohen's d effect size is 0.5, the researcher would need to add only four females to detect a sex effect.

Dr. Miller said that in many clinical trials, the percentage of men and women in the trial are given but only aggregate data are presented. She suggested that investigators instead present data by sex. Even if investigators have some statistical power issues, their data could help determine the number of individuals that should be added. Data also could be mined from existing large databases to gain insights into sex differences in disease presentation, outcome, mortality, and treatment effect without having to create another clinical study.

Dr. Michèle Ramsay said she was pleased to see an international trend toward data sharing by investigators, including the sharing of raw data. This allows analyses to be performed without having to use aggregated data. She added that it might help if NIH made this a requirement for funding. Dr. Stranger said it would be useful if NIH could help with sharing disaggregated summary statistics for genetics, as this had not always occurred. She added that the full set of summary statistics was rarely available by sex. She said that this would be a logical place for NIH to apply pressure and enforce the data sharing policy. Dr. Eugenia Trushina agreed and said NIH should consider developing a clear sharing statement for reviewers. Also, investigators submitting applications for grants should offer detailed explanations describing how they would share outcomes; they should not simply state generally that they will "share data." A specific question could be asked, such as "Are you going to upload data irrespective of outcomes?"

An attendee suggested that the Food and Drug Administration (FDA) should require that data from phase III clinical trials be disaggregated so researchers could study responses by sex and age. Dr. Miller said FDA already required drug reporting by age, sex, and ethnicity, but the data were not being crossed. FDA created a webpage for the latest approved drugs, called Drug Trials Snapshots (https://www.fda.gov/drugs/drug-approvals-and-databases/drug-trials-snapshots). The page provides information on side effects and relative effectiveness for males versus females. It might not always be as in-depth as needed, but it was a start. There was movement within FDA to improve that effort. A participant said clinicians were not using data from FDA when writing prescriptions or thinking about therapies; in other words, it was not being translated into patient treatment. Dr. Miller agreed that there was a gap in translation but said it was up to the medical community and professional societies within the medical community to push that information out.

Dr. Jayne Danska said that another important target audience for disaggregated data is journal editors. Some articles do not report the sex of animals used in the studies. At a minimum, they should be required to report husbandry conditions for animals, as well as the sex of the animals used. Dr. Miller said that there had been efforts to bring journal editors together, with mixed success. Some efforts were

carried out by the Society for Women's Health Research. A Canadian group developed the Sex and Gender Equity in Research (SAGER) guidelines. She added that some journals already required sex information. For example, the Endocrinology Society was requiring the sex of animals used in the research to appear in the titles of papers published. She said researchers could work within their professional societies and their societies' publications to support this kind of development of editorial policies. Moreover, seeing as many investigators are also reviewers, they have the "power of the pen" to reject papers that are not reporting on sex.

Dr. Emeran Mayer said some studies work well in multiple animal models but do not translate to humans. A cardiology investigator at UCLA was using a mouse diversity panel featuring 100 inbred mice with different genetic backgrounds. The investigator noticed that cardiac arrhythmia was arising in different genetic strains by different mechanisms. He asked what could be done to address this variation.

Dr. Trushina said there is no ideal mouse model for any disease. She said caloric restriction to prolong longevity and enhance health span was tested in about 30 mice. In one-third of the mice, caloric restriction had a detrimental effect, and they died. In another third, there was no effect. In the last third, caloric restriction had a positive effect on longevity. Dr. Trushina said her lab runs tests on four types of mice with different genetic backgrounds. For sufficient, efficient, and successful drug discovery programs, one needs animal models, human systems, translational applications, and scans of specific ion channels. Looking only at mice is not sufficient.

Dr. Hewitt asked whether individuals who had experience with study sections, reviews of applications for grants, or manuscript reviews perceived that sex as a biological variable was not fully understood. Dr. Sharon Elliot replied that in the grant reviews she worked on, the reviewers did not pay attention to SABV. She said that the Center for Scientific Review (CSR) had to do a better job of finding reviewers sensitive to this topic. Dr. McCarthy added that there should be more consistency, as there was significant variability across study sections. In the review sessions that she had attended, she had seen scientific review officers (SROs) giving very different instructions to reviewers, ranging from "nobody gets punished for not including females, just a warning" to "if they don't include both sexes, the grant is out." Dr. Hewitt said they were actively working on this policy with CSR. It was a concern to NIH that the policy was sometimes followed and sometimes not. NIH was taking this seriously to make sure that both the applicants and the review community could have the tools needed in this area. She thanked Dr. McCarthy for her feedback.

In addition, Dr. Hewitt asked how many of those present had challenged colleagues about their attitudes toward SABV. Only a few individuals raised their hands. She said she hoped participants would feel empowered to do so in the future. She added that they had seen applications for studying a single sex with appropriate justifications. "Cost" or "this is the way we've always done it" are not sufficient justifications, she said.

Dr. Danska said that Dr. Miller and other researchers had been in touch with the Canadian Institutes of Health Research (CIHR) with respect to reviewer education related to the importance of SABV. She added that they were working to bring together a "College of Reviewers," which would recruit using a set of uniform criteria. Canadian researchers developed some good online tools for education with respect to SABV. These will be phased in as a requirement for reviewers in all CIHR panels, irrespective of topic. Reviewers will be required to go through online training modules, and once training is completed, they should understand the role of sex and gender with respect to the panels they are on

and the subject matter they will review. She added that it would be nice to support similar learning module opportunities in the U.S.—or at least pilot them to determine whether they might be useful.

Dr. Miller replied that NIH was reworking its modules in a more integrative way, not necessarily by sex but by system. She said that she and her fellow researchers at the Mayo Clinic were using the Canadian online modules in their training program. Each free module is about 30 minutes long. The modules provide examples of grants and ask the trainee questions about how the grants address sex or gender. Researchers could promote the use of these modules by their laboratory trainees and try to embed them into other training programs within their institutions. Dr. Miller added that they were trying to embed them into the graduate school's ethics course because that course was required. Dr. Nieman noted that some grants required the investigators to complete some modules.

## *Closing Remarks*

***James M. Anderson, M.D., Ph.D., Director, Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI), Office of the Director, NIH***

Dr. James Anderson acknowledged that SABV has not always been a priority for investigators. He added that it can be a struggle for some researchers to understand how to implement SABV in their studies. Therefore, it is important to provide them with evidence to help them recognize that considering sex in experimental design and analyses is not only an NIH policy but a bedrock of valid science. He noted that the NIH Common Fund and ORWH jointly developed the supplemental awards that spurred many of the SABV studies presented during the workshop. This work resulted in compelling examples of the benefits of incorporating SABV into research, and he was confident that it would help investigators in the future.

Dr. Anderson closed the meeting by thanking all presenters for their work and the data they presented, which added to the body of evidence supporting the importance of SABV. He also thanked ORWH Director Janine A. Clayton, M.D., and NIH Office of Strategic Coordination Director Elizabeth Wilder, Ph.D., and their staff members for supporting the workshop.