Response to NCTR SAB Subcommittee Review of the Division of Biochemical Toxicology, dated 2 November 2021

We appreciate the time and effort spent by the NCTR SAB subcommittee in reviewing the Division of Biochemical Toxicology (DBT). Clearly much thought went into the preparation of the report. We believe the suggestions have already provided and will continue to provide guidance for the Division.

In preparing our reply, we have followed the outline of the review document.

General recommendations for the Division of Biochemical Toxicology

Comment: The DBT is a valuable resource for the NCTR, providing expertise to conduct both basic and applied research to evaluate biochemical mechanisms of toxicity of products relevant to the FDA. This strength is recognized within as well as outside of NCTR, as evidenced by the important collaborations established within NCTR and with other Centers (CDER, CBER, CDRH, CFSAN, CTP, CVM) and other federal institutes/agencies and universities. There is a need to establish priorities regarding research questions and strategies to address them. This requires leadership within the focus areas to bridge communications with other units and to assess the most pressing needs.

Response: DBT, perhaps to a greater extent than any other division at NCTR, conducts studies/research in direct support of requests made by FDA regulatory centers. Current examples include studies being conducted on cannabidiol, tattoo pigments, in vitro dermal penetration, brominated vegetable oils, perfluorinated alkyl substances, nattokinase/lumbrokinase, and nicotine. We have regular meetings with the regulatory centers (e.g., CFSAN and CTP) to report our progress and plan new studies. While we can and do set priorities of DBT-initiated studies, the SAB subcommittee needs to recognize that the priorities of the regulatory centers take precedence.

Comment: The DBT has experienced recent retirement of 3 key scientists. One was Dr. Jeffrey Fisher, the head of the computational modeling group. Before retirement, he had assembled an outstanding group of young scientists with expertise in modeling. This effort would be best served by appointment of a senior scientist, especially one who has safety assessment experience or to lead the program to develop its core competency and establish an integrated computational modeling-data collection program with other laboratories within the NCTR. Another option would be to provide opportunity for existing personnel to train with advisors in the regulatory programs to gain experience in safety. The two other individuals are Dr. Daniel Doerge and Dr. Mary Boudreau. The decision to replace these individuals is currently tied to future needs for pharmacokinetic studies and NTP funding issues. As mentioned in the

previous paragraph, some of the focus areas would benefit from more senior leadership, and it is recommended that the voids created by departure of Drs. Doerge and Boudreau be used as an opportunity to obtain that leadership.

Response: With regard to the retirement of Dr. Jeffrey Fisher, the Subcommittee recommended two potential pathways, recruit a senior scientist to lead the computational modeling group or provide additional training to existing personnel to gain experience in safety. The NCTR has had a long tradition of recruiting young scientists who have established very productive research programs. Examples include Drs. William Slikker, Tucker Patterson, Fred Kadlubar, Carl Cerniglia, Peter Fu, K. Barry Delclos, and Daniel Doerge, to mention a few. The recruitment of senior scientists has met with mixed success. Upon the retirement of Dr. Fisher, Dr. Annie Lumen stepped-in to lead the group. Unfortunately, in November 2021, Dr. Lumen left NCTR to take a position with a pharmaceutical company.

The computational model group currently consists of three staff fellows and three postdoctoral fellows. These individuals work together as a cohesive unit and have established productive collaborations with scientists in FDA regulatory centers and academic institutions. As such, we intend to follow the second of the two pathways recommended by the Subcommittee and provide opportunities for the existing personnel to gain more experience by training with senior advisors.

With regard to the retirements of Drs. Daniel Doerge and Mary Boudreau, we have continued to meet our commitments for the projects they were leading. Dr. Svitlana Shpyleva, an M.D./Ph.D., has completed the mouse tattoo experiments initiated by Dr. Boudreau and is in the process of expanding the scope of the work to include a tattoo study with minipigs. Dr. Frederick A. Beland has taken over the responsibility for completing the perfluorinated alkyl substance project that was being initiated by Dr. Doerge. Additional pharmacokinetic studies that focus on cannabidiol are being directed by Drs. Luísa Camacho and Qiangen Wu, while other pharmacokinetic studies are being led by Dr. Gonçalo Gamboa da Costa. As such, we believe we have the staff and resources to meet current and future needs.

Comment: Recent changes in the Interagency Agreement with NIEHS/DNTP stand to jeopardize maintenance of key infrastructure (facilities and personnel) necessary to conduct studies that support FDA Product Centers. It is recommended that a strategy be devised to prevent a disruption in service or a need to rebuild these capabilities.

Response: The comment is perhaps best addressed by NCTR's management, but we have recently been notified that through the actions of Drs. William Slikker, Tucker Patterson, and Gonçalo Gamboa da Costa that we will receive FDA funding to make up partially for the shortfall in NTP funding. This will contribute to ensuring that the

infrastructure will be maintained to conduct guideline studies requested by FDA regulatory centers.

Comment: It was mentioned both during the general SAB meeting and the Subcommittee Review that the DBT could take a leadership role in providing results from animal studies that could be used to evaluate the usefulness of alternative *in vitro* and *in silico* approaches that are being adopted. It is recommended that the DBT give this consideration.

Response: We agree with this recommendation and the funds mentioned in the response to the previous comment will be used to support this effort. For example, we have been having discussion with CDRH, CFSAN, and CVM about conducting in vitro-in vivo comparisons of skin irritation tests for safety assessment of FDA-regulated products.

Comment: Dermal studies conducted by the DBT are essential to expand knowledge of the extent of dermal absorption, and 3D-bioprinted human skin is an exciting new approach to evaluate skin absorption for product development and risk assessment purposes. Efforts to optimize this approach should continue.

Response: In vitro human dermal absorption studies are being completed with avobenzone and will be initiated with 1,4-dioxane. Dr. Luísa Camacho is leading studies to determine the strengths and weaknesses of a 3D-bioprinted human skin model. Toward this end, she has recruited a very talented postdoctoral fellow to assist in the studies. She is also interacting with scientists in Portugal who have developed an alternative human skin model that may help overcome some of the limitations identified with the 3D-bioprinted skin model currently under evaluation.

Comment: Efforts to develop methods using metabolically competent liver cells are viewed as valuable.

Response: We appreciate the Subcommittee's comment concerning this. Dr. Lei Guo continues to lead this effort. She and her team have recently expanded the work to include 3D models.

Comment: The epigenetics effort within the DBT would benefit from an expert in epigenetics and epigenetics data analysis. The goals of the individual projects, their novelty within the field and the link to the overall mission should be articulated.

Response: We are perplexed by the first part of this comment. Dr. Igor Pogribny has in excess of 260 publications, the majority of which deal with epigenetics, in particular related to liver toxicity and liver cancer. One of his epigenetics papers received the "2019-2020 Paper of the Year Award" in the journal *Toxicological Sciences*. Dr.

Pogribny's studies have been cited more than 13,000 times and he is a member of the Senior Biological Research Service, a category reserved for the most senior of FDA scientists. Members of his team, in particular Dr. Volodymyr Tryndyak, are very well versed in epigenetic data analysis. Dr. Beverly Lyn-Cook was recruited to NCTR by Dr. Lionel Poirier, one of the pioneers in the field of epigenetics. Dr. Lyn-Cook has continued to conduct studies that involve epigenetics, with a particular focus on breast and pancreatic cancer and lupus. Dr. George Hammons has maintained a part-time appointment at NCTR for many years and provides an interface to Philander Smith College, an historically black institution located in Little Rock, where he served as the Chairman of the Chemistry Department. Dr. Hammons has collaborated extensively with Dr. Lyn-Cook and is currently leading an investigation on the interaction between nanomaterials and epigenetics, with a focus on human lung cells.

During our presentations, we attempted to relate the importance of each of the studies and their link to the overall mission of the FDA. We apologize if we were not successful, but this may be due in part to the "virtual" format of the site visit, which was not as conducive to interactions as an "in-person" format.

Comment: There is the potential for the time and effort of the computational modeling group to be taken predominantly by projects in support of other Centers as opposed to research instigated within the DBT or NCTR. The DBT should decide strategically if this is the primary function of the computational modeling program.

Response: As noted above, a primary focus of the Division is to address requests made by FDA regulatory centers. This has also been a focus of the modeling group. An examination of the publication records of individuals in the modeling group (including Dr. Fisher) clearly indicates extensive interactions with scientists in FDA regulatory centers, as well as with other Federal agencies. We anticipate that this will continue.

Comment: The objectives of the studies being conducted by the computational modeling group should be aligned more closely with the scientific questions to be addressed.

Response: Two members of the modeling group, Dr. Miao Li and Dr. Kiara Fairman, are currently finishing projects initiated by Dr. Lumen. This is necessary to meet our commitments to the funding sources. Likewise, Dr. Darshan Mehta is completing a Center for Tobacco Products-funded project initiated by Dr. Fisher. He is also engaged in conversations with scientists at CFSAN regarding two projects, one that focuses on polyfluorinated alkyl substances (PFAS) and a second one that focuses on *C. elegans* and heavy metals.

Comments for individual topic areas

Research Focus Area 1 – COVID-19

Two projects were discussed under this research focus area, one by Dr. Camila Silva that focused on the detection of SARS-CoV-2 in wastewater and the second by Dr. Jia-Long Fang that focused on developing a flow cytometric analysis for anti-SARS-CoV-2 antibodies in human plasma.

Project 1: SARS-CoV-2 in wastewater

Comment: The Subcommittee stated this this approach could be a useful tool in the public health arsenal for SARS CoV-2 if appropriate epidemiological data could be obtained.

Response: Dr. Silva continues to make excellent progress on this project. She has monitored the presence of SARS-CoV-2 in wastewater in two metropolitan areas in Arkansas for nearly two years, which has allowed the investigation of the viral genomic dynamics during the major surges of COVID-19 cases. Using Next-Generation Sequencing, she confirmed the identity of SARS-CoV-2 and detected other pathogens in wastewater. She has implemented a genotyping RT-qPCR approach to screen these samples for SARS-CoV-2 mutations, and a new RNA sequencing method was successfully optimized for the identification of SARS-CoV-2 genetic variants. SARS-CoV-2 variants of concern (Alpha, Delta, and Omicron) responsible for the epidemic outbreaks in the area were identified in wastewater in all the study locations. In collaboration with epidemiologists from the Arkansas Department of Health, she has shown that the same SARS-CoV-2 variants of concern were found in COVID-19 patients from Arkansas during the same period. A manuscript describing these data is currently undergoing the internal review process at the Center.

Project 2: Flow cytometric analysis for anti-SARS-CoV-2 antibodies

Comment: The Subcommittee has a number of reservations about this project. Specifically, there was no articulation of the value in knowing the immunologic profile as opposed to the currently used antibody methods. There was no suggestion that a longer-term goal could be to use results from this method to understand pathogenesis or to direct therapy. Finally, the alignment of this project with the mission of the DBT was not clear.

Response: This project was the outgrowth of a project in which Dr. Fang developed a flow cytometric analysis for detecting anti-PEG antibodies in human plasma. At the beginning of the COVID-19 pandemic in early 2020, we wondered if we could use a similar approach to detect anti-SARS-CoV-2 antibodies. To date, 2 µm CML latex beads, with a low nonspecific protein binding, have been coupled covalently to purified recombinant SARS-CoV-2 subunits (S1A, RBD, S1, S2, and N subunit) and BSA (serving as blank beads). A flow cytometer assay has been developed with commercially available human anti-SARS-CoV-2 subunits antibody standards. Using this assay, the prevalence of anti-SARS-CoV-2 IgG, IgM, and IgA is being assessed in

the 200 human plasmas. When this project was initiated in early 2020, we had no idea of the subsequent magnitude of the scientific response to the COVID-19 pandemic. Based upon what we now know in 2022, upon completion of the current study, we plan to shift our efforts to other avenues of research.

Research Focus Area 2 – Dermal Studies

Two projects were discussed under this research focus area, one that dealt with the biodistribution and transplacental transfer of tattoo pigments in mice and the second being that dealt with percutaneous absorption.

Project 1: Tattoo pigment biodistribution

Comment: The Subcommittee was generally supportive of this study. A concern was raised that the mouse might not be the best animal model to adequately replicate pigment biodistribution in humans. The Subcommittee suggested that pigs or minipigs should be a considered. The Subcommittee also felt that a valuable extension for this project would be to determine what factors, including combinations of pigments, influence the distribution of these chemicals.

Response: This project was led by Dr. Mary Boudreau and, as indicated above, Dr. Boudreau retired in the Fall of 2021. As also indicated above, Dr. Svitlana Shpyleva has stepped-in to complete the project. Dr. Shpyleva has completed the radioactivity measurements for all of the tissue samples and is preparing the final report for the study.

While useful information was obtained with the SKH1 mouse model, this particular strain of mice (and probably mice in general) present a number of problems. For example, we were unable to apply the desired amount of tattoo pigment and the skin healing process in mice differs appreciable from that in humans. Given these limitations, we have initiated discussions with CFSAN to develop a new protocol that will use minipigs as the experimental model. This effort will be led by Dr. Shpyleva. Part of the new protocol will address the final recommendation made by the Subcommittee.

Project 2: Percutaneous absorption

Comment: The Subcommittee felt that the skin absorption studies are highly relevant since the safety and efficacy data can help FDA product centers in their regulatory decisions. The Subcommittee also noted that the potential of 3D-bioprinted human skin equivalents to provide reliable skin absorption data and serve as a tool to support the regulatory mission of FDA is novel.

Response: The percutaneous absorption studies being conducted by Dr. Luísa Camacho have two components: an investigation of the pharmacokinetics of cannabidiol (CBD) after dermal application to rats and an investigation into the utility of a 3D-bioprinted human skin model for assessing in vitro dermal absorption. With regard

to the first component, the rats have been successfully treated and liquid chromatography tandem mass spectral analyses are currently being conducted to quantify the levels of CBD and its metabolites 7-hydroxy-CBD and 7-carboxy-CBD. An analytical method that was further optimized to afford a higher sensitivity and improve the quantification of CBD and its major metabolites is being used. These data will provide information to CFSAN on the bioavailability of CBD upon topical application over a range of doses and cosmetic-relevant formulations.

Much progress has been made evaluating the performance of the 3D-bioprinted human skin for in vitro permeation testing. The data indicate that the batch-to-batch variability of the model is higher than desired, and that the barrier function is significantly lower than that of native human skin. Efforts continue to overcome these limitations and to improve further the model, including testing of support membranes with different pore sizes and optimization of the incubation/maturation times. Also, consideration is being given to expand the evaluations to include a newly developed alternative model of human skin that incorporates an inert polystyrene scaffold in the dermal-equivalent layer to support better differentiated epidermis and minimize contraction, a major contributor to the variability of the 3D-bioprinted human skin model. The Subcommittee commented on the need to compare the 3D-bioprinted skin absorption data with available in vivo human absorption data. We agree with this recommendation. This is one of the specific aims of the study, as stated in the approved protocol.

Research Focus Area 3 – Toxicological Assessments

Three projects were discussed under this research focus area, one that focused on cannabidiol and male reproductive toxicity, a second that dealt with metformin/glyburide and male reproductive toxicity, and a third that dealt with metabolically competent liver cells.

Project 1: Cannabidiol and male reproductive toxicity

Comment: The Subcommittee stated that the high quality of research in this focus area was evident. As indicated below, the Subcommittee did have some minor concerns with regard to this project.

Response: The focus of the project, which is being directed by Dr. Si Chen, is to evaluate the cytotoxicity of CBD and its major metabolites (7-hydroxy-CBD and 7-carboxy-CBD) in human and mouse Sertoli and Leydig cells. Dr. Chen and her postdoctoral fellow Dr. Yuxi Li have completed the evaluation with human and mouse Sertoli cells and the results have been published in *Food and Chemical Toxicology*. Similar experiments are currently being conducted with human and mouse Leydig cells.

The Subcommittee inquired about the rationale for investigating cytoskeleton reorganization. This endpoint was selected because the cytoskeleton protein F-actin is essential for important cellular functions, such as the motility and contraction of cells

during cell division. As such, it could be involved as an upstream event of CBD-induced inhibition of cellular proliferation.

The Subcommittee expressed concerns about the in vitro to in vivo linkage. In healthy volunteers consuming 1,500 mg CBD/day for seven consecutive days, the C_{max} of CBD in plasma ranged from 0.9 to 2.3 µM. Thus, we believe that concentrations of CBD (7 - 10 µM) used in the incubations with the human Sertoli cells approached clinically relevant human plasma concentrations.

The Subcommittee recommended that the potential molecular mechanisms of CBD-induced inhibition of proliferation in human Sertoli and Leydig cells be investigated further. This has been accomplished using RNA sequencing techniques and Drs. Chen and Li are currently preparing two manuscripts describing the results.

We agree with the Subcommittee's suggestions on using PBPK modeling to define the relationship between reproductive safety and CBD exposure and that a further investigation on CBD receptor-mediated effects would greatly contribute to the understanding of the safety of CBD. Dr. Chen is currently having discussions with colleagues at CFSAN regarding future directions for this project.

Project 2: Metformin/glyburide and male reproductive toxicity

Comment: This project was well received by the Subcommittee. The Subcommittee did feel that the inclusion of a pilot study to evaluate the effectiveness of the dosing strategy was important.

Response: At the time of the Subcommittee review, this study had not yet started. The experiment protocol was still being reviewed and was approved shortly after the meeting, with some design changes in the toxicity study in response to suggestions made by CDER scientists.

As noted above, the Subcommittee felt the inclusion of a pilot study was important and they were correct because some difficulties were encountered with respect to the formulation of glyburide formulation, breeding success, and the induction of glucose intolerance in the dams. As a consequence, a modified pilot study is going to be conducted to address the issues encountered. We anticipate initiating the developmental reproductive toxicity study later this year.

Project 3: Metabolically competent liver cells

Comment: The Subcommittee commented that this is an ambitious project that has potential for high impact. The Subcommittee encouraged the engagement of the pharmaceutical industry to use these platforms early in development. The

Subcommittee also felt that an important aspect to this research should be evaluating the comparability of CYP-expressing HepG2 cell model to currently used in vitro systems (including those under development) for regulatory decision-making.

Response: We appreciate the Subcommittee's strong support for this project. In order to engage the pharmaceutical industry, Dr. Guo and her team members intend to continue to present and promote their systems via scientific meetings, publications, and personal communication with scientists in the pharmaceutical sector. In addition, Dr. Guo and her colleagues plan to conduct comprehensive assessments of their CYP-expressing HepG2 cell model and currently used in vitro models, including primary human hepatocytes. The comparisons will include determining the levels of gene expression, protein levels, and enzymatic activity. Cytotoxic responses to liver toxicants will also be evaluated. Dr. Lei Guo and the team recently proposed to include 3D liver cell models as part of their drug-induced liver toxicity studies and these physiologically relevant models will also be used for comparative purposes.

Research Focus Area 4 – Epigenetics

Three projects were discussed under this research focus area, one that focused on non-alcoholic steatohepatitis, a second that dealt with nanomaterials, and a third that had two components, one that focused on triple negative breast cancer and the other that investigated systemic lupus erythematosus. The Subcommittee had major reservations regarding this focal area. We believe that we can address each of the concerns.

Project 1: Non-alcoholic steatohepatitis

Comment: Current findings from a study performed to identify phenotypic and epigenetic changes in NAFL-resistant and -prone mice were presented. Several studies have already been published on NASH and DNA methylation. It will be important to highlight what is new about this study and how it will contribute to our broader understanding that would enable better therapies or biomarkers.

Response: The main goals of this study are to (a) investigate the role of epigenetic disturbances in the molecular pathogenesis of NAFLD and its progression to NASH and NASH-related carcinogenesis and (b) identify epigenetic alterations that can be used to improve clinical diagnostic of NAFLD and be a target for drug development. To achieve these goals, Dr. Pogribny's laboratory used several human-relevant models of NAFLD, including a dietary choline- and folate-deficient model, a Stelic animal model, and a dietary high-fat and high-sucrose diet model. These studies were supported by an interagency agreement between the NCI and NCTR and by FDA extramural funding. Twelve peer-reviewed manuscripts describing DNA methylation, histone modification changes, alterations in the expression of protein coding and non-coding genes, molecular, cellular, and metabolic aberrations have been published. The current study is based on the findings of the previous experiments and focuses on uncovering of epigenetic changes responsible for the progression of the disease and the development

of NAFLD-related liver cancer and identification of epigenetic disease-associated alterations that can be used as diagnostic markers and pathways that could be targets for drug development for disease treatment and prevention.

Comment: The use of Collaborative Cross (CC) mice, a high-diversity mouse population, to investigate strain- and sex-related differences in susceptibility to NAFLD and its progression to early NASH is a strength in the study but it was unclear how the investigators planned to differentiate between susceptibility of differentially methylated regions (DMRs) vs those induced by specific diets (high fat/high sucrose, HF/HS) used in the experiment. The data need to be interpreted to address the main point of the study which is understanding predisposition to NAFLD/NASH.

Response: The Subcommittee brings up a very important point of how to distinguish differentially methylated regions (DMRs) associated with an increased susceptibility to the disease versus those induced by the high-fat and high-sucrose (HF/HS) diet that drive the development of the disease. We believe that the limited time allowed for the presentation and follow-up discussion did not allow this question to be addressed. We are planning to analyze DMRs in disease-resistant and disease-sensitive control mice to identify DMRs that may be associated with increased susceptibility to the NAFLD. The analysis of DMRs in disease-resistant and disease-sensitive mice fed the HF/HS diet will allow identification of DMRs associated with the development of NAFLD. In a recent article published in the journal *Epigenetics*, Dr. Pogribny's team identified several DMR-containing genes linked to the development of NAFLD.

Comment: Pathway analysis is useful but a more in-depth analysis of DMRs is important to decipher the meaning and impact of DMRs after HF/HS diets in mice and also male/female differences.

Response: We agree with the Subcommittee's statement about the importance of in-depth analysis of DMRs in addition to pathway analysis. As stated in the written documentation provided to the Subcommittee, Dr. Pogribny's team is using sophisticated techniques and data analysis tools, such as Methyl-Seq targeted DNA methylation next-generation sequencing and RNA-seq gene expression analysis. The data presented in Dr. Pogribny's presentation and in the recent *Epigenetics* article were obtained by employing these tools.

Comment: The lack of human samples is viewed as a problem, especially given previously published data. Different data measurements (RNA, methylation, etc.) need to be integrated. It was unclear which genetic measurements as stated would be studied. Only epigenetic changes are measured. In addition to liver tissue analysis, it could be useful to also measure other tissue types collected (e.g., muscle) from mice.

Response: We agree on the importance of studying molecular mechanisms of NAFLD, as well as identification of molecular and pathological drivers for stratification of the disease in humans. However, clinical and epidemiological cohorts are widely heterogeneous, and great difficulties exist with early diagnosis and disease staging

through non-invasive methods. To address this issue, Dr. Pogribny has established a collaboration with Dr. Arun J. Sanyal (Virginia Commonwealth University) to obtain human samples and conduct molecular analyses. Dr. Pogribny is planning to investigate several molecular mechanisms of NAFLD pathogenesis, in addition to epigenetic alterations. For example, a manuscript recently accepted by the *Journal of Nutritional Biochemistry* describes alterations of the fatty acid composition in Collaborative Cross mice fed a HF/HS diet. Similar molecular analyses will be conducted on other organs and tissues, such as kidney, muscles, and visceral adipocyte tissues.

Project 2: Nanomaterials

Comment: The Subcommittee felt that this project was well aligned with the mission of the FDA. The Subcommittee did recommend that it is important to address the potential impact of the epigenetic alterations. The Subcommittee also expressed concerns about the techniques used in the study, feeling that some were outdated.

Response: We agree with the Subcommittee's recommendation and a critical next step will to be assess functionality. Nonetheless, one of the ongoing challenges in this research area is demonstrating a causal link between the epigenetic event and an adverse outcome.

We believe that the PCR array approach being used continues to be appropriate to address the study objectives, as are the methods indicated by the Subcommittee. The methods were selected based upon the objectives of the study. The results of these methods formed the basis of a manuscript recently submitted for publication in *Nanotoxicology*.

Project 3: Triple negative breast cancer and systemic lupus erythematosus

Integration into FDA Mission

Comment: The goals of the research need to be better defined as they pertain to the mission. For example, how the specific epigenetic findings support the mission of the FDA and translate to improve human health should be articulated as opposed to "we need better biomarkers for …. lupus".

Response: We agree with the Subcommittee that epigenetics play a critical role in human health and disease. This project supports NCTR's mission to develop translational research approaches that provide the FDA with science- and data-based methods to improve public health. Due to the limited time allowed for the presentation, the relevance of the work to the FDA was not adequately expressed in the oral presentation to the Subcommittee.

Lupus is a priority area for FDA's Office of Minority Health and Equity and FDA's Office of Women's Health because of the lack of drugs for this disease and high incidence of

lupus in women, particularly African American women. Recently, the importance of epigenetic regulation of critical genes in the interferon pathway has been shown by Dr. Lyn-Cook's team and others. This pathway is currently being investigated as a target in a number of clinical trials using several epigenetic drugs. The high level of specific interferon gene expression regulated by DNA methylation in Dr. Lyn-Cook's epigenome-wide studies is relevant to FDA's mission because a number of biologics (e.g., antifrolumab) targeting this interferon pathway have shown promising results in patients with lupus who have a high interferon gene expression profile, as indicated in the written material provided to the Subcommittee.

Quality of Research

Comments: However, a major concern stems from the lack of strong epigenetics expertise in the division which can be noticed in the publication history. The lack of expertise was also evident from the data and study designs presented and from questioning during the presentations where there was insufficient information provided on the methodologies and analytical methods used to study DNA methylation or other epigenetic changes. The division would benefit from an expert in the field.

The program would also stand to benefit by having clearer and more focused research goals.

Response: We have responded to a similar comment made earlier in the Subcommittee report. To reiterate, Dr. Lyn-Cook has been conducting epigenetic research since the early 1990's, initially in collaboration with Dr. Lionel Poirier, one of the pioneers in the field who was the first to show the role of methylation in dietary restriction. Dr. Lyn-Cook is considered an expert in the field and works closely with other experts, including biostatisticians. An example of such a multidisciplinary group effort is the *Journal of Autoimmunity* publication by her laboratory reporting the findings of the lupus epigenome-wide study.

Project 3a: Triple negative breast cancer

Comment: Studying the potential for epigenetic therapies in the treatment of TNBC is valuable. One concern is that not enough due diligence was conducted to understand this space. Vorinostat and other histone deacetylase inhibitors have been widely studied in TNB and clinical trials have been conducted as well. Re-expression of ER and HER2 have been reported previously after vorinostat treatment in TNBC. These findings were not acknowledged in the study design presented. The study needs to define its novelty and improve scientific rigor given previous published data. Only cell lines studies are outlined which will offer very little in terms of impact**.**

Response: We appreciate the Subcommittee's comments. Given that much of the data had been published, detailed information was not presented at the Subcommittee meeting. References were presented in the written documentation provided to the Subcommittee.

Dr. Lyn-Cook has long had an interest in nutrigenomics and the interaction with anti-cancer drugs. The novelty of this study is the investigation of vorinostat effects on subtypes of TNBC and the effects of the dietary agent indole-3-carbinol. The presence or absence of the expression of ER, PR, or HER2 receptors is critical for therapy. Another finding is that vorinostat-induced re-expressed ER and PR led to the inhibition of cell growth and the sensitization of the cells to tamoxifen. A very important aspect of this study is the focus on TNBC cell lines from African American patients. Understanding the effects of HDAC inhibitors, such as vorinostat, in subtypes of TNBC continues to need further investigation, particularly in African Americans who die at a very young age due to aggressive subtypes of this disease. Furthermore, the study indicated that vorinostat showed specificity for HDAC7, which has a role in cancer stem cells known to play a role in resistant and relapsing breast cancer. Currently, there are several clinical trials with vorinostat in combination with other drugs that show some promising results, while others show high toxicity levels.

Project 3b: Systemic lupus erythematosus

Comment: A better understanding of how and why the selected markers were chosen should be demonstrated. Simply filtering beta values in BeadStudio is inadequate and incorrect for selecting regions even with a Bonferroni-adjusted value.

Response: We appreciate the Subcommittee's comments. The analysis in epigenome-wide study was conducted by an expert statistician and the results of the study were published in the *Journal of Autoimmunity*.

Research Focus Area 5 – Computational Modeling

Five projects were discussed under this research focus area – one that focused on a PBPK model for nicotine in humans, a second on a generic perinatal life stage PBPK modeling platform, a third on an artificial intelligence (AI)-driven virtual pregnant woman modeling suite, a fourth on various computational modeling collaborations to support FDA Centers, and a fifth on an approach to select complex mixtures for toxicological evaluations. The Subcommittee noted that the computational modeling work performed in the Division was of high quality.

Project 1: Multi-pathway PBPK model for nicotine in humans

Comment: This project was well received by the Subcommittee. The focus of this project was to support the FDA Center for Tobacco Product's (CTP) request to develop a multi-pathway PBPK model for predicting nicotine pharmacokinetics in humans. The modeling group had recently published a paper describing a PBPK model for nicotine in nonhuman primates. This project was designed to extend the PBPK model predictions to humans. The Subcommittee did express a concern that the model outputs were not clearly linked to the toxic effects or addictive properties of nicotine.

Response: The Subcommittee has raised a valid point; nonetheless, it should be noted that the project objectives and outcomes were determined by the research needs of the CTP. We were requested to develop a PBPK model for nicotine to assist the reviewers at CTP to predict the internal tissue dosimetry of nicotine and its major metabolites (cotinine and trans-3'-hydroxycotinine) after exposure to various types of tobacco products, such as cigarettes, cigars, electronic nicotine delivery systems, and smokeless tobacco. The eventual intent is for this PBPK model to serve as a tool for CTP reviewers to determine internal dose metrics relevant to addiction endpoints and toxic effects of nicotine in humans.

Comment: Another inquiry made by the Subcommittee was how the dose-response relationship established in experimental animals will be extrapolated to humans with only the human model.

Response: This, too, is an important point. We would like to note that, due to time limitations, only the human PBPK modeling results were presented at the meeting. We have a similar project ongoing (that is also funded by CTP) for developing a PBPK model for nicotine and its metabolites in rats using the pharmacokinetic datasets that were generated in-house by exposing Sprague-Dawley rats to four different nicotine dose levels via three exposure routes (intravenous injection, oral gavage, and nose-only inhalation). The findings from this PBPK model for rats will be used to bridge the gap between the dose-response relationship observed in experimental animals and humans.

Project 2: First-generation in-house PBPK model-based tool

Comment: The proposed in-house perinatal life stage PBPK model, potentially with a graphical user interface, will address the current challenges related to model transparency and accessibility. This tool has the potential to be the core product of the computational modeling program in DBT to support various Centers in the FDA when internal dosimetry predictions are needed for pregnant and pediatric populations.

Response: We appreciate the positive comment from the Subcommittee. The perinatal life stage PBPK model is a powerful tool for both research and regulatory purposes. Currently, we have collaborators from FDA regulatory centers to ensure that the research can help support FDA reviewers using PBPK models for decision-making.

Comment: Given that some of the projects focus on developing generic modeling tools, more in silico tools (such as the Gastroplus' ADMET predictor) that can be used to generate physio-chemical properties and ADME parameter values for PBPK modeling can be explored, especially the open-source options. Also, cheminformatic tools (such as structural similarity analysis and classification algorithms) that can be used to generate early ADME predictions can be incorporated into the various modeling suites that are being developed in the program.

Response: We appreciate the Subcommittee's suggestion and agree that the incorporation of open-source chemoinformatic tools for the prediction of physicochemical properties and parameter values will be helpful in establishing a suite for the perinatal-relevant PBPK models. Some open-source tools in R have been used to support our current PBPK modeling research, such as the "httk" package developed by EPA to help predict tissue-to-plasma partition coefficients, and the "webchem" package to retrieve physicochemical properties from PubChem. We are also aware of other open-source in-silico tools that are helpful in overcoming data gaps for new chemicals. These tools include the OPERA and QSAR Toolbox for QSAR/QSPR models and CompTox Chemicals Dashboard for chemical properties and chemical-relevant literature.

Comment: All research projects have the potential to significantly contribute to the FDA's public health mission, if the modeling work can clearly link to human relevant exposures, human relevant toxicity/drug efficacy, or both.

Response: We appreciate this comment. The case studies related to the perinatal in-house PBPK modeling tool are based on drugs currently used off-label at different perinatal life stages. Pharmacodynamic or toxicodynamic differences in perinatal-relevant life stages compared to healthy adults are also considered to adjust dosage for these special populations in case studies.

Comment: These modeling efforts will have a much higher impact if the investigators have a clear and specific scope/purpose and testable hypothesis prior to deciding which model design is the most appropriate for the intended purpose.

Response: We agree with the Subcommittee's comment. To construct the in-house PBPK model-based tool, we use different case studies for model development and validation. All the drugs for case studies were carefully selected based on several criteria, such as current concerns about use during perinatal life stages, lack of dosage guidance for off-label use for these life stages, and previously reported perinatal clinical PK data. The main goal of the project is to support dose adjustments for perinatal life stages to ensure drug safety and efficacy.

Comment: One potential concern is that their success in these collaborations may lead to many smaller modeling projects that take up too much of the investigators' time. If the primary function is to develop modeling tools to fill an important gap (such as pregnancy PK modeling), then the investigators should be given adequate resources, including time, to contribute to the development of a core product.

Response: As noted previously, a primary focus of the Division is to address requests made by the regulatory centers. This has also been a focus of the modeling group and an examination of the modeling group's publication record clearly indicates extensive

interactions with scientists in FDA regulatory centers, as well as with other Federal agencies. We anticipate that this will continue. In addition, the computational modeling group is encouraged to develop innovative proposals and research ideas and is provided with adequate resources to contribute to the development of a core product. One such example includes utilizing both the in vivo and in vitro capabilities within the Division to develop the pregnancy PBPK model and fill important data gaps in regulatory science.

Project 3: Artificial intelligence (AI)-driven virtual pregnant woman modeling suite

Comment: The description of the study design requires more detail. It is unclear whether artificial intelligence and machine learning (AI/ML) are proposed to inform the selection of influential model parameters, based on specific drug class, to be included in a PBPK model; or to predict some PK data (e.g., AUC, plasma conc over time?) or some PK changes (e.g., fold changes in AUC?) during pregnancy for either a specific drug or a specific drug class.

Response: We appreciate the Subcommittee's comments. Our current approach is to use AI/ML to select the most influential model parameters for a specific drug class to be included in a PBPK model. We are leveraging the extended clearance classification system (ECCS) for drugs to estimate a drug's clearance mechanism depending on its specific drug class. We will then utilize AI/ML approaches to help determine certain clearance mechanisms that should be included or excluded in the PBPK model based on the broader classification system. Next, we will examine the sensitivity of model parameters by evaluating their impact on changes in pharmacokinetic endpoints, such as AUC, C_{max}, and T_{max}. The model parameters that prove to be most sensitive will be evaluated further based on previously published data that are available for the specific drug class. This will be conducted by automating the data search process and using AI/ML approaches with specific keywords for the drug class of interest. This approach will help to increase our confidence in the values used for the sensitive model parameters, which in turn will increase our confidence in the overall model. We will also explore the use of AI/ML approaches to automate the process of specifying physicochemical properties of drugs and running the sensitivity analysis for the PBPK model simulations.

Comment: It is also unclear if the purpose is to replace PBPK models with an AI/ML informed "virtual pregnant women platform" or to support the development of PBPK models.

Response: The purpose of this model is not to replace PBPK models with an AI/ML-informed "virtual pregnant women platform", but instead to support and inform the development of PBPK models. The AI/ML model can support the development of PBPK models by serving as a tool to refine the values of sensitive model parameters by leveraging information from the drug classification systems and automating the data search processes. A trained AI/ML model can assist in addressing the data gaps that

exist when developing a pregnancy model (e.g., in enzyme activity, transporter activity, and other ADME processes) with a greater level of confidence.

Comment: Finally, this pregnant woman modeling suite is proposed to solve the problem that developing a pregnancy PBPK model for one computer at a time is time- and labor-intensive. This objective can also be achieved with the proposed in house PBPK model-based tool. A joint effort between the two projects could result in a better product.

Response: We agree with the Subcommittee's comment that a joint effort between the pregnancy PBPK project and the AI/ML project would be of value. The incorporation of AI/ML could contribute to the pregnancy PBPK project by helping address uncertain model parameters (e.g., unknown activity changes of transporters or enzymes during pregnancy). We are currently working together to achieve the AI/ML portion of the project and we have enlisted other AI/ML experts within NCTR to help in this research effort.

Project 4: Various computational modeling collaborations to support FDA Centers

Comment: The five projects showcase the program's capability to offer a wide range of computational modeling support to various FDA Centers and initiatives.

Response: We appreciate the Subcommittee's positive comment. The collaborative projects help us maintain and establish connections within FDA regulatory centers and other federal agencies. For example, Dr. Li is actively involved in Toxicology in the 21st century (Tox21) initiative, which has expanded our modeling capability to apply in vitro to in vivo tools to support risk assessment. Dr. Fairman is involved in in vitro to in vivo extrapolations as part of the Botanical Safety Consortium. Dr. Mehta is engaged in discussions with investigators at CFSAN on projects regarding PFAS and the use of *C. elegans* in toxicological assessments.

Project 5: An approach to select complex mixtures for toxicological evaluations

Comment: It is unclear how this project is related to computational modeling. If computational modeling approaches are used to merge analytical data, the details of such approaches are not provided in the presentation.

Response: We agree with the Subcommittee's comment: this analysis is, strictly speaking, not a computational modeling approach. Nonetheless, we are using multivariate statistics, including principal component analysis (PCA) and hierarchical cluster analysis (HCA), to visualize the inherent structure of data generated by analytical techniques. Briefly, analytical techniques like ^{1}H NMR and UHPLC-DAD-ESI-HRMS generate large amounts of data for a sample mixture. The volume of spectral information generated from multiple sample mixtures can be difficult to interpret when

the chemical constituents are unknown. To improve the interpretability of models, ^{1}H NMR and UHPLC-DAD-ESI-HRMS data will be processed individually through unsupervised analysis (PCA and HCA) and then the data will be integrated and analyzed through Multi-block analysis to reveal common trends. This approach is expected to help guide the selection of complex mixtures as test articles of toxicological studies.