

**Endocrine Disruptor Methods Advisory Committee
Plenary Teleconference Meeting
August 2, 2005**

Meeting Summary

On August 2, 2005, the U.S. Environmental Protection Agency (EPA) convened the second meeting of the Endocrine Disruptor Methods Advisory Committee (EDMVAC) by conference call. The meeting objectives included:

1. Review the EPA's interest and role in validating the 15-day Adult Intact Male Assay (in rats);
2. Review results from previous studies conducted by industry, laboratories and others;
3. Present study design for inter-laboratory validation;
4. Justify selection of key aspects of the study design; and
5. Solicit commentary and advice from EDMVAC members regarding previous studies and proposed inter-laboratory validation study.

Copies of presentation slides and other materials distributed at the meeting may be obtained by contacting Jane Smith at smith.jane-scott@epa.gov or 202/564-8476. Many of the materials are also available on the EPA website at <http://www.epa.gov/scipoly/oscpendo/>. EPA established an administrative record for this meeting under docket control number OPPT-2005-0037. The official public docket is the collection of materials available for the public at the EPA Docket Center, Rm. B102-Reading Room, EPA West, 1301 Constitution Ave., N.W. Washington, DC. The EPA Docket Center is open from 8:30 a.m. to 4:30 p.m., Monday through Friday, excluding legal holidays. The EPA Docket Center Reading Room telephone number is (202) 566-1744 and the telephone number for the OPPT Docket, which is located in EPA Docket Center, is (202) 566-0280.

I. Welcome, Introductions, Agenda Review, and Ground Rules

Jane Smith, Designated Federal Official, EPA Endocrine Disruptors Screening Program (EDSP), opened the conference call by briefly explaining that the meeting was conducted in accordance to the Federal Advisory Committee Act (FACA). She explained that background materials for the meeting were available on the EPA website and in the docket. She noted that written comments on meeting topics should be submitted to the electronic docket or to Dr. Juliana Birkhoff, senior mediator with RESOLVE and facilitator of the meeting. Ms. Smith also covered the specific materials all participants should have on hand during the call.

EDMVAC members and conference call participants introduced themselves.

Dr. Gerald LeBlanc, EDMVAC Chair, welcomed EDMVAC members and went over the purpose and focus of the meeting. He noted that this particular meeting was intended to focus on the single topic of the 15-day Adult Intact Male Assay and asked that the Committee provide advice to EPA on that assay alone. Noting limited time and current EPA

needs, Dr. LeBlanc cautioned the Committee to avoid the following topics:

- 1) debating EPA's definition of the validation process, and
- 2) judging the overall acceptability of the assay relative to other assays.

Dr. LeBlanc explained that the focus of the meeting should be on reviewing previous and more recent results and to consider the design for the next steps toward the inter-laboratory validation phase. EDMVAC members were asked to offer advice on what else might need to be done to optimize the protocol.

A participant noted a discrepancy between Dr. LeBlanc's guidance and the discussion questions outlined in the meeting agenda. The Committee discussed whether the assay was still in the pre-validation phase or in preparation for the validation phase. After a brief discussion, Dr. LeBlanc clarified that the EDMVAC was providing advice on next steps in the validation of the assay and that this next step was not necessarily the final step in validation. The next steps are aimed at further optimizing the assay protocol and establishing reproducibility of results across laboratories.

Discussion of the demarcation between pre-validation and validation was tabled until a later meeting when the EDMVAC will have a chance to review the EPA position paper on validation as it pertains to the Endocrine Disruptor Screening Program.

Dr. Juliana Birkhoff reviewed the ground rules and agenda for the meeting. She reiterated that participants could submit written comments to her or the electronic docket after the meeting.

II. EPA's Role in the Adult Male Assay

Gary Timm and Dr. Don Bergfelt, EPA Endocrine Disruptors Program, provided an overview of EPA's role in the 15-day Adult Intact Male Assay. Dr. Bergfelt referred Committee Members to the "Introduction to Background Materials" document to read about the specifics of EPA's role. That document also outlined the timeline for the next steps in validation for the Committee to discuss. Dr. Bergfelt noted that the assay is being considered as an alternative assay in the Tier 1 screening battery. EPA would like to commence testing before the end of the current contract in January 2006 to prevent future delays. Mr. Timm and Dr. Bergfelt acknowledged the limited amount of time for EDMVAC members to absorb the most recent information on the assay. They asked that members please give their best effort and thinking on all aspects of the assay to date, but specifically on the two key discussion questions in spite of the time constraints.

III. Industry's Role in the Adult Male Assay

Dr. Rick Becker, American Chemistry Council, provided an overview of industry's role in the 15-day Adult Intact Male Assay. Dr. Becker reviewed some of the details about the assay. It is a screening mode of action assay (MOA) included in the 1998 EDSTAC report as part of the alternative Tier 1 screening battery. He reminded members and participants that

Tier 1 screening assays are intended to be short-term, inexpensive, easy to execute and should detect endocrine disrupting effects. Dr. Becker explained that the assay was designed to replace the Hershberger and Pubertal Assays. Dr. Becker noted that the MOA screening approach has many advantages including an intact reproductive system and a series of hormonal endpoints. He added that the approach was envisioned as reducing the number of animals used in the screening tier.

Dr. Becker pointed out that OECD has included the 15-day Adult Intact Male Assay as one of its multi-modal assays. He noted that work on the assay has been led by the Haskell Laboratory of Dupont. He also mentioned that the peer reviewed publications on the assay were listed in the meeting background materials. Dr. Becker noted that more than 30 chemicals have been assessed using the assay.

Dr. Becker emphasized that it is important to note both the strengths and weaknesses of the assay. He said the assay presents no serious logistical problems, but that serum analysis of hormones could be difficult and is not routine for laboratories. Dr. Becker stated that the key strength of the assay is that it provides a fingerprint or pattern for hormonal and organ weight profiles as compared to positive controls. Histopathology endpoints are optional but are typically done for the testis, epididymus and thyroid. The assay can identify potential endocrine-active compounds and characterize the MOA. He noted that many studies have been done using the assay and mentioned a few other laboratories that have worked with it (for example, BASF, RTI, Syngenta, Dow, and WIL).

Dr. Becker asked EDMVAC members to please not compare the specificity or reproducibility of the assay to other assays at this time. He noted that in a mechanistic screen the assay was shown to be responsive to thyroid active agents. He suggested the assay could potentially be an alternative to the Frog Metamorphosis Assay. He also highlighted that the discussion was not about the last stage of validation, but an extension of ongoing work to further optimize the protocol and gather data on cross-laboratory transferability.

IV. Industry's Overview of the Adult Male Assay

Presentation

Mr. John O'Connor, Dupont Haskell Laboratory for Health and Environmental Sciences, provided an overview of industry's work on the 15-day Adult Intact Male Assay to date [as indicated above, copies of this slide presentation and background materials can be obtained from the docket and the website]. Mr. O'Connor compared the EDSTAC-recommended Assays for identifying endocrine-active compounds (EACs) to the alternative Tier 1 screening batteries. He noted that his presentation would focus on an Alternative Screening Battery 1 including the 15-day Adult Intact Male Assay. He outlined the desirable attributes of a screening assay:

- Reliable identification of known EACs or estrogen, androgenic and thyrotropic (EAT) activity;
- Predictive (known EACs are identified for the mode of action);
- Sensitive (low false-negatives);

- Quick (short-term);
- Cost effective; and
- Minimize animal usage.

Mr. O'Connor summarized the pre-validation study design for the Adult Intact Male Assay used at Dupont and all other laboratories. He highlighted a set of specific study design issues for EDMVAC members as follows:

- Whether oral dosing is the most relevant route;
- The best way to select the dose level;
- Whether to use adult or immature animals;
- If a 2-week duration is appropriate; and
- How to deal with strain sensitivity differences.

Mr. O'Connor reviewed and interpreted data on the effect of dietary restrictions on organ weights, serum hormones, and thyroid hormones in Sprague-Dawley rats. He presented data from case studies comparing the sensitivity of organ weight, serum hormone, and thyroid hormone in immature rats to the sensitivity of mature rats. He suggested that the organ weights of immature rats were more sensitive than adult rats, while serum hormone measurements in mature rats were more sensitive than immature rats. Mr. O'Connor stated these correlations and noted that research is ongoing to clarify sensitivity differences between immature and mature rats. He posed that a combination of a hormonal battery and organ weight measurements can result in an estimation of the mode of action.

Mr. O'Connor presented a list of the EACs that have been examined in the Adult Intact Male Assay and a list of the EACs examined in the Pubertal Assays. He suggested that data comparisons focus on compounds that have been run in both of these assays as well as others. Mr. O'Connor summarized the advantages of using the Assay in the Tier 1 Screening Battery, as follows:

- It is a comprehensive MOA screen:
 - Capable of evaluating several different MOA in a single assay -- by measuring mechanistic endpoints (androgen, estrogen and thyroid agonists/antagonists; steroid hormone synthesis (aromatase & steroidogenesis);
 - Tier I with intact male provides MOA "profile" to focus direction of any further testing; and
 - Reduces the number of animals needed for Tier I.
- Maintains an intact endocrine system:
 - Design allows integration of new endpoints if desired.

He concluded with a description of the protocol design for the next round of studies to evaluate the transferability of the protocol and reproducibility of results.

Clarification Questions

A participant asked if the same dietary restriction study was performed on both immature and mature rats. Mr. O'Connor replied that the data presented were old and that the same studies were not performed on both. Another participant asked what diet was used in the studies and

inquired if there was an effort to consider phytoestrogens. Mr. O'Connor stated the rats were fed standard Purina 5002 diet.

A participant asked Mr. O'Connor if he thought the assay protocol transferred well between laboratories in studies conducted to date. Mr. O'Connor responded that the two-week gavage study had been transferable so far. He noted that difficulties had arisen with the serum hormone results.

V. Questions and Discussions

EDMVAC members were asked to provide feedback to EPA on the following discussion questions:

1. **Protocol optimization and transferability:** Considering the number of chemicals and latest efforts that have been run in the various industrial and contract laboratories in which expected results have been documented, does the EDMVAC agree that the protocol is ready for inter-laboratory validation? If not, what additional information would be necessary to proceed?
2. **Chemical selection:** Considering the number and types of chemicals that have been selected to challenge the assay over several modes of action and compare with previous results, does the EDMVAC agree with the selection of the number of laboratories and chemicals for inter-laboratory validation? If not, what should be considered?

Question 1 - Discussion

EDMVAC Chair, Dr. Gerald LeBlanc began by framing the discussion for EDMVAC members. He explained that the Committee should decide whether the assay protocol is sufficient to proceed with the next step of validation. He encouraged the Committee to point out problems that need to be addressed and offer advice on how to improve the protocol.

A participant highlighted hormone analysis as a strength of the 15-day Adult Intact Male Assay. It could also be a weakness because of the extent of biological variability and the requirement for technical expertise. The participant asked how blood samples are collected. Mr. O'Connor informed the Committee that after the last dose at necropsy, the animals are transported in their cages to the necropsy room one hour before blood samples are collected (via the *vena cava*) two to three hours after the final dosing to minimize stress due to cage transport that may alter hormone concentrations. The participant asked for clarification of the rationale behind the statistics for the hormone analysis. Mr. O'Connor explained that power calculations were done and that an *n* of 15 animals per treatment group is a sufficient number of animals to ensure an ability to detect weaker acting agents. Statistics were mentioned as a concern.

A participant pointed out that the results for linuron were not necessarily different from the

controls with respect to the MTD raising a question about transferability of the protocol. Mr. O'Connor acknowledged that the data for linuron are questionable, but that the results were not necessarily negative.

A participant asked what strains were used. Mr. O'Connor stated the strain was Sprague-Dawley. He acknowledged that there are likely to be strain sensitivity differences for different endpoints. He noted that this is a concern for the Adult Intact Male Assay and any other *in vivo* assay.

A participant inquired about what degree of effort is required to carry out the assay, noting that cost is not a good measure. Mr. O'Connor agreed cost is not a good measure. He stated that information about time requirements could be produced.

A participant asked if anesthesia is used in the protocol because it can affect organ weight and create variability. Mr. O'Connor explained that different forms of anesthesia were tried, but that CO₂ anesthesia was used for all studies. He noted that animals were moved to the necropsy area after final dosing and held there for one hour before sacrifice to minimize stress due to cage transplant. Blood was collected two to three hours after final dosing.

A participant asked what dose selection procedures were used in the protocol and whether they will be examined as part of the inter-laboratory effort. The participant noted that dose selection tends to be one of the greatest factors of variability across laboratories. Mr. O'Connor responded that to date, dose levels were given to the laboratories for data comparison purposes. He did suggest the point be considered by the EDMVAC. The participant said that even if dose levels were standardized, research would be required to determine dose selection variability across laboratories.

There were different opinions among the participants as to whether a dose-range finding study should be considered as part of validation.

A participant raised concern about the diet being used in the protocol. He cited that phytoestrogens have been documented in the literature in association with the diet. He suggested a diet with a controlled level of phytoestrogens be prescribed in the protocol to prevent the perpetuation of variation.

There were different opinions among the participants as to whether a diet low in phytoestrogens be considered in the Adult Intact Male Assay protocol.

The participant asked if consideration had been given to the selection of a positive control. He suggested at least one positive control be used to test the system to ensure a negative result is negative. Mr. O'Connor questioned which positive control would you select. He had developed "fingerprints" for many chemicals thought to be positive for comparison to "unknowns". There were different opinions among the participants as to whether a positive control be considered in the Adult Intact Male Assay protocol.

A participant asked for clarification about whether the assay will be run on mature or

immature rats. Mr. O'Connor explained that the plan was to use the adult rats. He highlighted that the data suggests that adult rats are satisfactory to moving ahead. He noted that there were small differences, but not to a degree to justify switching to immature rats. The participant asked whether there are endpoint "fingerprints" to use in validation studies which allow data to be interpreted in a consistent way. Mr. O'Connor explained there is a preliminary pattern of "fingerprints" with which to work.

A participant expressed ongoing concern about differences in sensitivity with organ weight and hormone levels. Another participant agreed there is a significant difference between measuring organ weights and measuring hormones. The participant stated that part of the protocol optimization process should be to evaluate how reliable hormone measurements are across laboratories.

There were no objections among the participants to move forward with the inter-laboratory phase.

Question 2 Discussion

A participant asked about the basis of the decision for evaluating study chemicals for the 15-day Adult Intact Male Assay in only two laboratories. John O'Connor noted that the design proposed in the "Rationale for Laboratory and Chemical Selection" [as indicated above, background materials are available on the website and in the docket] was only a proposed plan and was up for discussion. Dr. Bergfelt noted that EPA has limited resources under the current contract to conduct the next phase of testing and affirmed the tentativeness of the design presented and for the EDMVAC to discuss alternative designs. Mr. O'Connor framed the question as being about whether to test more chemicals with less modes of action or fewer chemicals with more modes of action.

A participant suggested a positive control be run in all four laboratories to develop criteria for what constitutes a good study. He also suggested running the same three chemicals in three different laboratories. This increases the chance of identifying sources of variation across laboratories. Another participant concurred that chemicals should be tested in at least three laboratories. He stated that if the method is not proven reproducible than whatever is found in isolated instances does not apply to developing a regulatory framework.

A few EDMVAC members commented on what the strength of a positive control should be. A few members suggested using a weak-acting positive control. Another participant noted that two of the chemicals selected were weak-acting and good choices. Multiple members supported the idea of using more laboratories to ensure optimization of the protocol so that variability might be minimized during full-blown validation. Of the members that commented, there was general agreement that fewer chemicals should be tested in more laboratories, and that weak acting positives should be the focus of evaluations. One participant did not consider Ketoconazole to be a weak acting chemical, and consequently should not be used in the study.

A participant recommended against the use of the Purina 5002 diet in the protocol. He

suggested using a low-phytoestrogen diet. Another participant disagreed, stating that the level of phytoestrogens in the Purina 5002 diet was not significant enough to affect adult rats.

A participant argued that laboratories should be given set dose levels because they are necessary to be able to compare RIA's.

There were several significant informal recommendations by the participants to the proposed protocol but not all were agreed upon by the participants.

Summary by EDMVAC Chair

EDMVAC Chair Gerald LeBlanc summarized the key points of discussion by the members:

1. Blood Collection– should be stated in the protocol;
2. Statistical Analyses – should be more robust and the analytical statistics method should be specified in the protocol;
3. Type of Anesthesia – must be specified in the protocol; do not leave that variable up to the laboratories;
4. Dose Selection – in this proposed study, the EPA should determine now the pros and cons of setting standard dose levels;
5. Number of Laboratories – should be as many as possible, more than two optimum; the group was aware of resource constraints, but noted that more laboratories would provide more information;
6. Number of Chemicals - limited by cost, 3 is optimum but may be prohibitive, 2 will be acceptable; testing more chemicals would be optimum, but if industry has to choose between more laboratories or more chemicals, it should choose more laboratories;
7. Positive Controls – there is a need for positive controls in the study to establish acceptance criteria;
8. Phytoestrogens in Diet - the protocol should use a low estrogen diet; and one person noted that this was of negligible significance for adult male rat.

V. Public Comments

Dr. Earl Grey, EPA/ORD

This [adult male] assay does not address strain and sensitivity. The chemical DDE is negative for hypospadias in this assay. In the Hershberger Assay, DDE is positive for anti-androgen effects. Linuron in four other studies was demonstrated to be anti-androgenic but failed to give expected diagnostic profile for anti-androgenicity in the adult male. These DDE and Linuron chemicals are not producing hormonal profiles because they are not interacting in the HPG axis.

The immature animal is more sensitive than adult animal and so we find that in comparing

vinclozolin in the ED profile responds at lower doses and more dramatically than the adult male. He may provide additional comments, see EPA Docket No. OPPT-2005-0037.

Dr. Sue Marty, DOW Chemical

Dr. Sue Marty stated that she had experience working with the Hershberger, Pubertal and Adult Male Assays. She pointed out that all of the assays have strengths and weaknesses. Dr. Marty suggested industry and EPA should move ahead with the Adult Intact Male Assay so that it can eventually be compared with the other assays.

VI. Next Steps

Dr. Juliana Birkhoff explained that all comments made during the meeting would be captured in the meeting summary. She also reminded participants to submit further comments in writing to RESOLVE or Jane Smith at EPA.

Jane Smith, DFO and EPA Endocrine Disruptors Screening Program, announced that the October EDMVAC meeting was canceled due to a lack of completed data on the next topics up for discussion by the EDMVAC.

The meeting adjourned at 2 o'clock.

Attachments

- A. EDMVAC Members in Attendance
- B. Additional Member Comment – Dr. Edward Orlando
- C. Additional Member Comment – Dr. Nancy Kim
- D. Additional Member Comment – Dr. Paul Foster
- E. Additional Member Comment – Dr. Gerald LeBlanc
- F. Additional Member Comment – Dr. Shane Snyder
- G. Additional Member Comment – Dr. James Owens

Attachment A

EDMVAC Members in Attendance

Gerald LeBlanc, Chair
Mildred Christian
Robert Combes
Rodger Curren
Peter DeFur
Anne Fairbrother
Paul Foster
David Hattan
William Kelce
Sean Kennedy
Nancy Kim
Steven Levine
Edward Orlando
James Owens
Shane Snyder
James Stevens
William Stokes
Glen Van Der Kraak

Attachment B

08/02/2005 02:23 PM

cc Jane-Scott Smith/DC/USEPA/US@EPA, Gerald LeBlanc
<ga_leblanc@ncsu.edu>, "Orlando, Edward F" <eforlando@smcm.edu>, eorlando@fau.edu

Subject: Follow-up comments to the August 2, 2005 Conference Call

Dear Juliana,

Please include the following follow-up comment to your summary.

Dr. Earl Gray made the point that pp-DDE and Linuron have effects on ASG structures when administered at the time of sexual differentiation.

Flutamide and not HO-flutamide appears to have been used by the investigators in Dr. O'Conner's presentation, where he showed no differences between immature and adult male rat ASG.

Hydroxy-flutamide is the more potent metabolite of flutamide, and I am wondering if they examined immature vs. male effects from exposure to this compound?

+++++

My overall comments are that I would like to see 3 labs (min) and 3 compounds (to test three MOA) as part of the inter-laboratory (pre-) validation. Also, I think standardizing both the compound concentration and RIA protocol seem necessary to assess variability in the MOA measured among labs and that allowing the concentration to be chosen by the individual labs would be introducing additional sources of variation.

Sincerely,
Ed Orlando

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After August 15, 2005:
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Attachment C

From: Nancy K. Kim [nkk01@health.state.ny.us]
Sent: Tuesday, August 02, 2005 6:11 PM
To: Juliana Birkhoff; GA_LeBlanc@ncsu.edu
Subject: Re: EDMVAC Conference Call Evaluation and Next Steps

Hi Juliana and Jerry,

I had one comment that I wanted to make about the issue of whether or not the labs should determine the dosing levels or whether the dosing levels should be set for all the labs in the protocol. I share some of Willy's concerns, but decided that EPA should really figure out what the pros and cons are of setting the dosing levels or of having the individual labs determine the levels. I came up with this question for EPA although other wording may be better.

If the protocol calls for the individual labs to set the dosing levels and if the dose selection is not consistent among the laboratories, what, if any thing will be lost in terms of timing, information gained, costs, etc?

My thought was that we don't know what is going to happen if the individual labs set the doses. If everyone sets the same dose, it won't matter and you've gained another step along the way. If they are set at very different levels, how great will the losses be, if any, in terms of drawing conclusions from the study?

I think if EPA can work out the pros and cons of either outcome that should help them in making a decision about which approach might be better.

Nancy Kim

Attachment D

From: Foster, Paul (NIH/NIEHS) [foster2@niehs.nih.gov]
Sent: Wednesday, August 03, 2005 7:12 AM
To: Juliana Birkhoff
Cc: 'GA_LeBlanc@ncsu.edu'
Subject: RE: EDMVAC Conference Call Evaluation and Next Steps
Juliana,

I believe that I had the opportunity to make most of the comments that I wanted to make at the meeting which were briefly:

1. Since DDE did not work in the SD (CD) rat what strain is being proposed for the study?
Answer - SD

2. An examination of the RTI data, based on the criteria presented by Dr. O'Connor, did NOT show a positive response for linuron (a weak antiandrogen) i.e. it did not give the correct pattern of response (high LH, low T) and 3 of the 4 dose levels exceeded the "MTD" of < 10% decrease in terminal body weight compared to the control. A - Dr Connor agreed that Linuron has been "problematical" even in his hands.

3. Can we get some idea of the level of effort required to conduct this study? It is complex, employs histopathology and a thorough examination of the animals' endocrine profile. This, together with the number of animals employed (15 per group) could make this cost-prohibitive as a tier 1 screen.

4. The mode of anesthesia is an important consideration in making single point hormone determinations since many anesthetics interfere with steroid and pituitary hormone levels. Rapid anesthesia is also important to minimize stress.

5. If budget is an issue for the next stage, then better to have more labs and limit the examination to weaker acting agents (Phenobarbital and linuron were suggested). Obviously more labs and more agents would be preferable.

6. How are dose levels selected for these studies? Do we need an examination at some time of the ability of a lab to examine agents "blind" and therefore have to conduct dose setting activities.

As we were progressing through the conference call I became more uneasy about the use of this assay, but am not sure that this has reached the level of a fatal flaw - but almost. Dr O'Connor's presentation spent some time about where this protocol would fit in within the tier 1 battery - as a potential alternative to the Hershberger or male pubertal assays. A primary function of these two assays is to detect, with some surety, weak acting antiandrogens. This assay has manifestly failed to do this in its failure to adequately detect DDE and Linuron, the only weak acting antiandrogens in the available list. Both these agents give reasonable positive signals in the Hershberger assay and both have been shown to

be teratogenic to the developing reproductive system when administered in utero. This lack of sensitivity to a critical group of agents the assay would be specifically charged with detecting is a very serious concern that I don't believe the proposed study would reconcile.

Regards

Paul Foster

Attachment E

From: Gerald LeBlanc [ga_leblanc@ncsu.edu]
Sent: Tuesday, August 02, 2005 8:23 PM
To: Juliana Birkhoff
Cc: Smith.Jane-Scott@epamail.epa.gov; Angela Agosto; Bradford Spangler
Subject: Re: Language for Meeting Summary and Next Steps

Hi Juliana,

Here is my summary of member recommendations.

The following protocol modifications/improvements were suggested by Committee Members. No dissent was voiced concerning the first three items.

- 1) The source of blood sampled from rats should be stated in the protocol.
- 2) Statistical analyses of hormone results needs to be carefully considered with a suitably robust method recommended in the protocol.
- 3) Type of anesthesia must be specified.

In addition, some members felt strongly about the following three items, though dissent was expressed by one member.

- 4) Labs should establish their own dosing levels as this may prove to be a major source of variation among participating labs. If so, this source of variation must be identified and reconciled. The dissenter felt that this would significantly increase costs and animal usage in the validation process and could add a layer of variability that shouldn't be considered at this time.
- 5) Low estrogen diets should be required. The dissenter felt that this concern was not warranted when using adult male rats.
- 6) Each assay should be accompanied by a positive control. This treatment should consist of a weak-acting chemical. The positive control would facilitate the development of acceptance criteria. I'm not sure why dissent was expressed about this...probably the added cost involved.

Finally, the Committee seemed to concur that, in this next stage in pre-validation, the lab/chemical matrix should be weighed in favor of labs over chemicals.

Jerry

Attachment F

Hello Juliana & Gerry:

I enjoyed the conference call and thought it went well over all. Your > suggestions for improving the call are excellent, and I especially > encourage a roll call of the EDMVAC members by RESOLVE. I am highly > impressed by the Intact Adult Male Rat Assay. While more data are > certainly needed to confirm appropriate sensitivity, I believe the data > gathered thus far are promising. I believe this assay offers many > advantages over the individual assays it could replace. I strongly > recommend that EPA continue to support the development/refinement of the Intact Adult Male Rat Assay and move expeditiously towards validating this particular assay.

Best Regards,

Shane

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Attachment G

1. Overview of comments from James Owens

These comments are broken into the following areas:

- Summary Answers to the Agency's Two Questions.
- Two strategic questions about validation concerns to the Agency that apply to several assays at this time, including the 15-day male:
 - The need to carefully craft and state the purpose of the assay, noting that "endocrine disruption" has not been, and is unlikely to be defined by the Agency.
 - Thoughts on the need for a "prediction model" for validation and interpretative guidance for a regulatory guideline.
- Areas where I concur with expressed points of other EDMVAC members that I heard during the teleconference.
- Areas where I don't concur with expressed points of other EDMVAC members during the teleconference.
- Other thoughts that emerged after the teleconference

2. Summary Answers to the Agency's Two Questions

- A. **Question: Protocol optimization and transferability:** Considering the number of chemicals and latest efforts that have been run in the various industrial and contract laboratories in which expected results have been documented, does the EDMVAC agree that the protocol is ready for inter-laboratory validation? If not, what additional information would be necessary to proceed?

Answer: The previous black and white distinctions between prevalidation and validation can be questioned. Given the considerable resources required for validation, a single, all-or-nothing study is not wise for more complex assays such as the 15-day male or the pubertal assays. A series of staged studies or phases is far more appropriate for managing these validation studies, so that any questions can be identified and resolved, and the risk of losing these considerable resources is reduced. Thus, using previous approaches, some will argue that this is only "pre-validation." As noted during the teleconference, I don't support such a strict black and white construction and consider this proposal to be a transition into validation.

With that introduction, this transition plan is basically correct. The 15-day male has:

- a considerable history of use in the single DuPont lab,
- a plausible and relevant basis with the tissues and hormonal battery to clearly address a number of mechanisms of concern (e.g., estrogen-androgen-thyroid or EAT),
- demonstrated capability to address a number of EAT chemicals, both potent and weak
- some evidence of transferability between laboratories,

- the rat is an appropriate model consistent with higher tier developmental and reproductive assays,
- many endpoints such as organ and tissue weights and histopathology are widely used and not in question,
- the laboratory animal husbandry conditions are widely used, and
- this assay does afford a significant reduction in overall resources, including a reduction in the number of animals employed.

B. Question: Chemical selection: Considering the number and types of chemicals that have been selected to challenge the assay over several modes of action and compare with previous results, does the EDMVAC agree with the selection of the number of laboratories and chemicals for inter-laboratory validation? If not, what should be considered?

Answer: The classes of chemicals are appropriate for this transition study. They focus on the androgens, steroidogenesis, and thyroid toxicants. As the 15-day male would be paired with the uterotrophic bioassay addressing androgens, these are the true target areas for this assay.

The suggestion of phenobarbital and linuron for the thyroid and androgen areas are appropriate; these are weakly potent and should sufficiently challenge the performance of the assay for these mechanisms.

The suggestion of Ketoconazole needs to be reviewed. It is a relatively potent compound, and a compound of weaker potency would be preferable to challenge the performance of the assay for the steroidogenesis mechanism.

3. DETAILED COMMENTS

3.1 The purpose of an assay

My point is to raise a potential Catch-22 situation that may confront several assays. At this time, several assays (e.g., the 15-day male, the pubertal, the fish screen) are being presented as “screens for endocrine disruption.” However, the Agency has consistently refused to define what constitutes endocrine disruption in the biological terms that would be needed for an assays intended purpose. If this proposed purpose cannot be clearly defined, including which chemicals should be positive and which negative, the implications are severe. This means, almost inherently, that such an assay cannot be clearly validated since a fundamental criteria for validation cannot be met, as the purpose is not clear. Therefore, I strongly urge the Agency to begin to consider how to list in a clear mechanistic way, consistent with their policy needs and with the biology, what the purpose of each assay may be. An example for though would be similar to the left hand column in slide 7 of 45 of John O’Connor’s presentation. This clearly lists the intended mechanisms that the assay should detect, by inference which mechanisms it is not, and one can use this list to declare and select both positive and negative substances.

3.2 Thoughts on prediction model

Previously, validation programs have dealt with assays that use only one or a handful of measurements. They were also intended to replace other assays. Thus, the prediction model was indeed necessary in such cases to demonstrate concurrence between the new assay and the assay to be replaced. Due to the limited number of endpoints or measurements, the construction of such a direct prediction model was not a difficult or even an insurmountable task. As we engage the validation of more complex, multi-purpose and, therefore, not just multiple measurements, but measurements with different toxicological characteristics and connected to different or multiple mechanisms, difficulties with the older prediction model arise. Again, as with slide 7 of 45 of John O'Connor's presentation, we are now seeking to establish which profile or fingerprint a test chemical may present, AND we interpret particular profiles as shown in slide 7 to represent particular mechanisms. It is through these toxicological profiles and their interpretation that many current assays will "predict," and, ultimately, achieve their purpose. Again, the assays appear in my opinion to include the 15-day male, the pubertal, the fish screen, and possibly the tadpole metamorphosis assay.

Therefore, as with the 15-day male, the Agency needs to begin:

- to construct and vet with the EDMVAC such profiles for several assays;
- to utilize these profiles to recommend positive and negative test substances; and
- to set up criteria for the success or failure of the assays to detect positive compounds (or yield false negatives) or to indicate negative compounds (or yield false positives).

This will provide a much clearer and, hopefully, less debatable, means for the EDMVAC to make recommendations on the utility and performance of the assays. It should also then provide a means for the EDMVAC recommendations to be transparent and credible with most stakeholders as well as the Agency.

3.3 Areas where I concur with expressed points of other EDMVAC members.

3.3.1 Importance of the Hormonal Battery to the 15-day Male Assay

I concur with the points of John O'Connor during the presentations and member observations during the teleconference. The 15-day Male heavily depends upon the robustness of its hormonal battery to deliver a clear mechanistic profile or fingerprint. Therefore, one clear focus area of this transition work should be to examine the ability of the labs to perform the profile and the robustness of that profile. As I note later in section 3.5.2, this has implications well beyond the 15-day male and the exercise should then have increased value for the Agency and EDMVAC. This involves several key areas in the protocol

3.3.2 Protocol Features

At least three protocol features need to be examined for the 15-day Male and for other assays as outlined below.

3.3.2.1 Animal Handling

The literature (e.g., Dohler, which I have referenced in previous FACA comments) indicates that many of the hormones have a circadian cycle and are affected by stress. The question about stress seems particularly true for the thyroid hormones. Therefore, consulting with the DuPont lab, the EPA group of Cooper et al who have worked on the pubertals, and others

such as EDMVAC member Mildred Christian of Argus, it appears to be essential that the time for necropsy be carefully planned and standardized as well as methods for handling the animals with a minimum of stress.

3.3.2.2 Method of Euthanasia

There is evidence that the method of euthanasia such as choice of anesthesia can induce stress and potential induce variation in some hormones. Again, with consultation, the method of euthanasia should be reviewed by experts, again emphasizing the thyroid hormones, in order to lead to common procedures amongst the labs in this transition study.

3.3.2.3 Method of Blood Sampling

I also encourage a similar examination of the blood sampling procedure, and the handling of the blood sample in order to reduce variations.

3.3.2.3 Hormonal Methods and Existing Laboratory Data

The analytical methods for the hormones should be reviewed for:

- what is the level of experience in the laboratory (how often are these analyses performed, are they routine or not?);
- what are the actual methods, i.e., identify the kits and their suppliers and review the SOPs amongst the labs. I'm not advocating strict standardization at this time, but a careful review of differences so that these can be investigated if the results indicate such a review is necessary;
- have the labs identified particular events or procedures that reduce variability, and so on so that these points are considered; and
- what are the historical baselines for values and variability amongst the labs with two purposes in mind 1) together with experience and SOPs to evaluate possible sources of variability and 2) the common use of such baselines to understand and interpret the results of particular individual studies.

3.4 Areas where I don't concur with expressed points of other EDMVAC members.

There are areas where I disagree with comments from other EDMVAC members, and the Agency needs to understand both points of view.

3.4.1 Diet and Phytoestrogens

Comments originating from studies with immature female mice continue to arise concerning the laboratory diets. As noted in my April presentation, food intake drives the actual ingested dose of phytoestrogens. These doses vary by species and by age, so that the mouse ingested dose is greater for the same diet as the food consumption is higher and the immature dose is greater than the young adult for the same reason. Calculations suggest that it is implausible that the adult rat is vulnerable. The data generated by the uterotrophic validation on diet; toxicogenomic data specifically studying whether there was the induction of estrogen genes in the female rat using diets such as the PMI 5002, see Naciff et al. (2004) *Env. Health Pers.* 112:1519–1526; and data from both Thigpen (mouse) and Ashby (rat) on the influence of dietary caloric intake should all collectively put to rest the hypothesis that these diets are not acceptable for the rat. In fact, the data indicate that they are acceptable. In

addition, the Agency has the pubertal assay database, most of which I believe has been performed with the 5002 or similar diets, and there are data from the OECD with the TG 407 studies and the Hershberger studies which also show no impact of the diet. Therefore, I see no need to pursue different diets.

3.4.2 Positive Controls in All Studies

To open with a rhetorical question: Does the Agency require positive control groups in a cancer study, in a developmental and reproductive two-gen, in a 91-day general tox study, or other major studies? No, for two reasons:

1. these have multiple endpoints and need to assess different outcomes simultaneously – so there is no clear rationale to choose a positive for an unknown compound, and
2. the significant increase in resources and animals for a given study, and cumulatively the impact could only be described as enormous.

The 15-day Male, the pubertals, the in utero-lactational, the tadpole metamorphosis, and the fish assay are all intended to address multiple mechanisms/outcomes and indeed the additional cost and animal usage would be significant.

In the uterotrophic program, we did observe a decreased response with the lower of two doses with the positive control (ethinyl estradiol) in labs that also had problems with weak substances, and this was related to the control uterine weight being high and less statistically powerful for small increases in uterine weight. These comprised about 5% of the overall results. In no other case, was there a problem with the positive control values. Therefore, the long term course would be to set acceptance criteria for the control uterine weights, not to require positive controls in all experiments.

Similarly, in the Hershberger, the positive control in the antiandrogens experiments, a dose of flutamide, has not indicated problems. There appears to be no long-term need.

Therefore, the Agency needs to make two decisions:

1. Is there a true justification and value for positive controls **in the transition study?**
2. How does one then choose a positive control for “all” mechanisms when dealing with an unknown, so that its relevance and use for data acceptance of other mechanisms and negatives is rational?

3.4.3 Individual Laboratory Dose Setting

Again, let me reiterate this is a transition study. The foremost needs at this point are to understand the inherent robustness of the assay itself with several key mechanisms.

There is first a philosophical difference to address. Just what are the boundaries of validation? I think there would be concurrence that we are examining the robustness and reproducibility of an assay, so that examining the assay and possible lab effects (contributions to variability such as a difference in tissue dissection and handling proficiency, the reading of slides by different pathologists, and differences in hormonal methodologies) are a necessary component of a validation study. I'm not sure that the effect of a purely laboratory judgement about the doses to be used is what we need to examine.

We acknowledge that in practice, difference in data and professional judgement are likely to lead to somewhat different doses in different laboratories. There is, however, general guidance for selecting doses, such as the need for the top dose 1) to yield either a positive effect for one or more endpoints or to demonstrate a maximum tolerated dose such as >10% difference in body weights with the vehicle control or 2) to be a limit dose of 1000 mg/kg/d. There is also general guidance on dose intervals, such as not to exceed an order of magnitude between doses. As a result, in practice, I not aware of situations where on a routine basis doses in laboratories done for the same purpose are radically different. I would like examples from the advocates of this position to show that there is a realistic, common problem to be addressed.

If we should come to agreement on the need for dose selection, I think that this activity is clearly the last stage of a validation program. I continue to argue for a progressive validation program for complex, multi-endpoint, multi-purpose assays like the 15-day Male. One should first explore its inherent reproducibility and the utility of those measurements to yield consistent and usable profiles with different mechanisms where weakly potent compounds are administered. Only when these are found to be acceptable, should studies allowing dose selection differences be considered. Thus, it is not appropriate at this time to consider individual laboratory dose selection issues.

3.5 Other Thoughts After the Teleconference

A more strategic view of the thyroid hormones has implications both for their use in several assays, but also for the evolution and practice of validation. Allow me to follow up on these two points to assist the Agency in the overall management of their program, meaning beyond the management of individual assays (pardon the analogy, don't manage the individual, separate silos, manage the whole farm).

3.5.1 Sensitivity to Thyroid Toxicants – the Male Rat

The male rat appears to be the most sensitive model for thyroid toxicants. Due to thyroid tumors in this species and sex, there is an extensive literature showing that 1) the hormones are not bound by a specific serum protein carrier, 2) this induces a very high rate of turnover (serum half-life) compared to most other species, and 3) this places the thyroid under threat of consistent pituitary stimulation leading to tumors in the long-term. For these same reasons, this model should be rapidly sensitive to thyroid toxicants. Therefore, work with this model and the thyroid hormones are of inherent value.

3.5.2 Importance of the Hormonal Battery to Other Assays

In this regard, the thyroid hormones are a feature in several other assays including the pubertals, potential additions to the multi-gen, the enhanced TG 407, the in utero-lactational, and even the tadpole. The same fundamental circadian, stress, necropsy timing, blood sampling, and laboratory measurement proficiency all apply. That is, for endpoints like the thyroid hormones, one confronts a situation not of validating an individual protocol, but validating a set of endpoints across several protocols. Again, a situation where the previous theory and limited practice of validation not confronts and must deal with something entire new as it evolves.

Therefore, I strongly encourage the Agency to think of the thyroid hormones as a cross cutting set of endpoints and measures, where the data from different assays should be pooled and taken into account during protocol writing and evaluations. I also would not take the approach that a complete and repetitive set of work on the thyroid hormones is necessary for each individual assay so that work is duplicated and amount of resources and numbers of animal are unnecessarily increased.