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TITLE: Mechanisms of Epileptogenesis and Circuit Dysfunction in a Mouse Model of TSC

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CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX

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14. ABSTRACT
Epilepsy in Tuberous Sclerosis Complex (TSC) is often resistant to traditional anti-epileptic drugs and to mTOR inhibitors. The structure and function of a brain circuit early after the onset of epilepsy may be different from that late in the course of epilepsy when seizures are drug resistant. Structure and function rely on gene expression. If we can compare gene expression of neurons at these early and late time points after the development of epilepsy, including when seizures are drug-resistant, we may be able to identify important genes and proteins involved in drug-resistant seizures. This knowledge may not only reveal underlying mechanisms of drug-resistance but may guide the development of more effective treatments. We are using a mouse model which exhibits the same seizure and drug resistance as TSC patients. In this first year of study, we found that mTOR pathway is reactivated which parallels the return of epileptic seizure and despite the continuous presence of the mTOR inhibitor RAD001.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

Epilepsy in Tuberous Sclerosis Complex (TSC) is often resistant to traditional anti-epileptic drugs and to mTOR inhibitors. We understand very little about how brain circuits in TSC become epileptic or drug-resistant in part because fluctuations in gene expression during the development of epilepsy present a moving target. It is not known if the gene expression status of epileptic brain circuits after epilepsy onset is involved in generating seizures or if it is a secondary consequence of seizure activity. The structure and function of a brain circuit early after the onset of epilepsy may be different from that late in the course of epilepsy when seizures are drug resistant. Structure and function rely on gene expression. If we can compare gene expression of neurons at these early and late time points after the development of epilepsy, including when seizures are drug-resistant, we may be able to identify important genes and proteins involved in drug-resistant seizures. It is difficult to tease apart the various components of a neuron's function and structure responsible for epilepsy or drug resistance in human tissue as we do not have the tissue before seizure development as control. The TSC mouse model we are using in the proposed studies exhibits severe epilepsy and responds to mTOR inhibitor, a common treatment for TSC patients. However, just like in TSC patients, mTOR inhibitors in this mouse are transiently effective but seizures re-emerge even when starting treatment very early in the life and after re-emergence are drug resistant. The seizure suppression and its subsequent resurgence in this mouse model provide an excellent opportunity to explore neuronal circuit deficits and gene expression alterations associated with drug resistance. Our goal is to understand the mechanisms underlying drug resistant seizures in TSC and thereby gain insights into novel therapeutic targets that would potentially be disease modifying for this aspect of the TSC phenotype.

2. KEYWORDSTSC
Drug-resistance
mTOR inhibitor
Differentially expressed genes
Epilepsy
Circuit behavior

3. ACCOMPLISHMENTS:
Major Goals

Specific Aim 1: To decipher characteristics of the seizure-prone circuit and associated molecular signature in NEX-*Tsc2* KO mice at the onset of epileptiform activity early in life.	**Timeline**	**% complete**	**Date Completed**
Major Task 1. vEEG analysis of epileptiform activity in KO mice to guide the microcircuit and molecular experiments Number of animals required = **30**	Months		
Subtask 1: Monitor EEG seizure phenotypes of Tsc2 deficiency • NEX-*Tsc*2 wildtype (WT) and knockout (KO) animals will be used. N=15 per genotype • EEG electrode implantation at P8 • vEEG activity and track epileptiform and seizure activity starting at postnatal day (P) P8 • Data analysis	22-27	50%	
Local IRB/IACUC Approval	1	100%	
HRPO/ACURO Approval	1-4	100%	
Colony building	5-8	100%	On going to maintain the colonies
Major Task 2 **Assessment of mTOR activity levels in KO and WT mice** Number of animals required = Will use animals from Major Tasks 1 post-EEG recording			
Subtask 1: Analysis of protein expression of the mTOR signaling pathway • WT and KO from Major Task 1 at P12 will be used • Western blot analysis • Immunohistochemistry	22-24	40%	

Major Task 3 **To identify synaptic connections and record electrophysiological properties of the neurons during early onset of epileptiform activity** Number of animals required = Will use animals from Major Tasks 1 post-EEG recording			
Subtask 1: Microcircuit analysis • Brain slices will be obtained from WT and KO mice from Major Task 2 at P12 will be used • Simultaneous whole-cell recordings of up to 8 neurons from brain slices • Morphological reconstructions and analysis	23-27	8%	
Subtask 2: scRNA sequencing with Patch-seq • The isolated RNA from above patched cells will be used for Patch-seq • RNA-seq data analysis	23-27	5%	
Specific Aim 2: To evaluate characteristics of the seizure-prone circuit and molecular signatures associated with mTOR inhibitor resistance in NEX-*Tsc2* KO mice.			
Major Task1 **Monitor epileptiform activity for emergence of seizures in KO mice with chronic RAD001 treatment** Number of animals required = **90**			

Subtask 1: EEG monitoring during RAD001 treatment to identify epileptiform and seizure activity • WT and KO animals treated with vehicle or RAD001 (6mg/kg every 2 days) starting on P8 • EEG electrode implantation at P8 • vEEG activity and track epileptiform and seizure activity –to identify pre-and post SZ animals (P40 and P50) • Data analysis	9-22	50%	
Major Task 2 **Assessment of mTOR activity levels in KO and WT mice treated with RAD001** Number of animals required = Will use animals from Major Tasks 1 post-EEG recording			
Subtask 1: Analysis of protein expression • WT and knockout KO animals from Major Task 1. at the pre-and post-seizure time points will be used (P40 and P50) • Western blot analysis • Immunohistochemistry	9-19	40%	
Major Task 3 **Determine the microcircuitry changes and cell type-specific transcriptomic signatures in pre- and post-development of RAD001-resistance in KO mice** Number of animals required = Will use animals from Major Tasks 1 post-EEG recording			

Subtask 1: Microcircuit analysis in animals seizure resistance vs. seizure onset determined in above subtask 1. • Brain slices will be obtained from WT and KO animals from Major Task 1 at the pre-and post-seizure time points (P40 and P50) • Simultaneous whole-cell recordings of up to 8 neurons from brain slices • Morphological reconstructions and analysis	10-22	0%	
Subtask 2: scRNA sequencing with Patch-seq • The isolated RNA from Subtask 1 above patched cells will be used for Patch-seq • RNA-seq data analysis	10-22	0%	
Milestone(s) Achieved: Manuscript writing and submission	28-36	0%	First manuscript in preparation

What was accomplished under these goals:

Specific Aim 1: To decipher characteristics of the seizure-prone circuit and associated molecular signature in NEX-*Tsc2* KO mice at the onset of epileptiform activity early in life.

Major Task 1. vEEG analysis of epileptiform activity in KO mice to guide the microcircuit and molecular experiments. We are completing the characterization of mTOR, seizure, and epileptiform activity in the Tsc2 KO mouse during early development. Determining the course of epilepsy in the Tsc2 KO will guide microcircuit analysis by providing optimal timepoints. Below are spectral analysis carried out on EEGs from Tsc2 KO mice during early postnatal development (Fig. 1 and 2). We have used 51 animals for EEG recording and tissue collection (3/group WT Veh, WT RAD, KO Veh, KO RAD from ages P8, P16, P25, P35, and P45). No tissue can be collected from KO Veh >P16 due to premature death. Tissue for immunohistochemistry has been fixed, post-processing is pending.

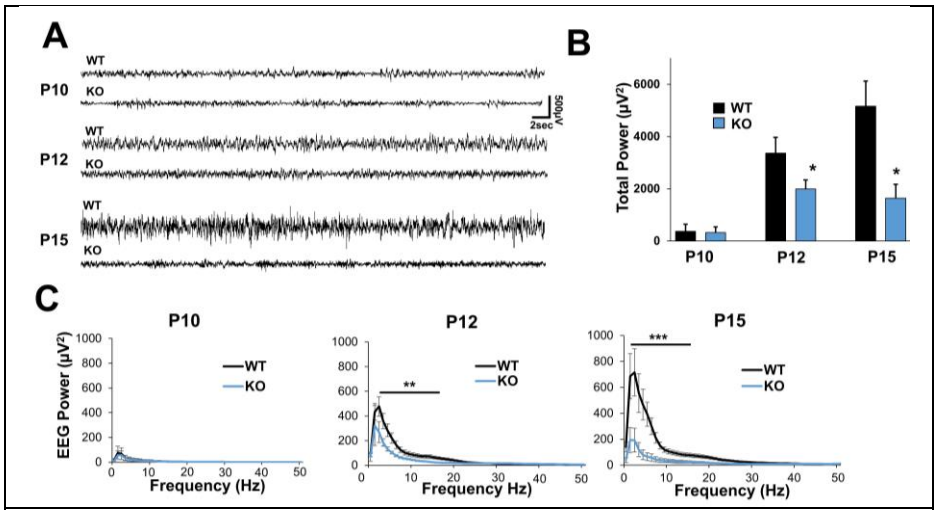

Figure 1. Tsc2 KO mice exhibit developmental deficits in EEG power. A) Sample EEG traces from WT and KO mice during early development. Deficits in total B) and spectral C) EEG power appear at postnatal day 12 and continue up to postnatal day 15.

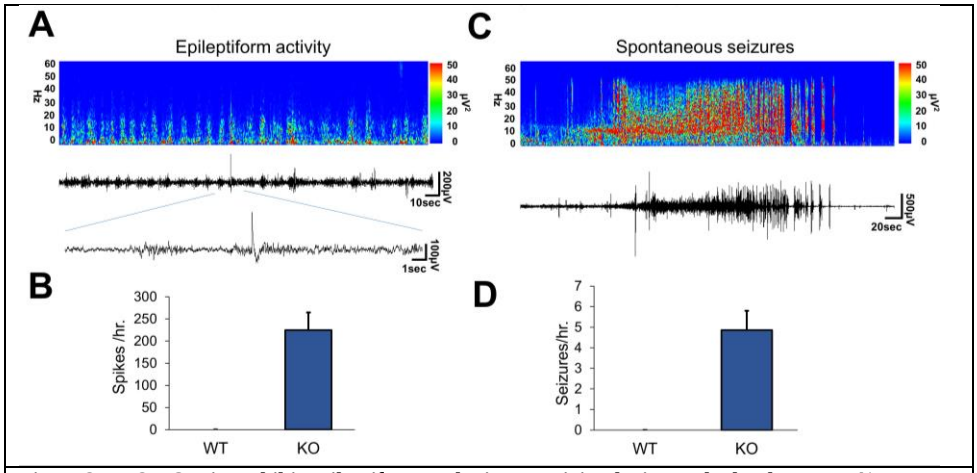

Figure 2. Tsc2 KO mice exhibit epileptiform and seizure activity during early development. A) Heat map and sample EEG trace showing spike activity in a Tsc2 KO mouse. B) Summary of average spike frequency in Tsc2 KO mice. C) Heat map and sample EEG trace showing a seizure in Tsc2 KO mouse. D) Graph of average seizure frequency in Tsc2 KO mice.

Major Task 2. Assessment of mTOR activity levels in KO and WT mice. mTOR dysregulation is at the core of TSC pathophysiology. Therefore, we are determining the time course of mTOR pathway dysregulation in the Tsc2 KO mouse at various time points early in development. These findings may better guide microcircuit analysis by providing optimal developmental time points. We have collected tissue from P8, P12, and P16 naïve mice (3/group WT and Tsc2 KO). Figure 3 shows western blot from brain tissue harvested from P16 Tsc2 KO mice.

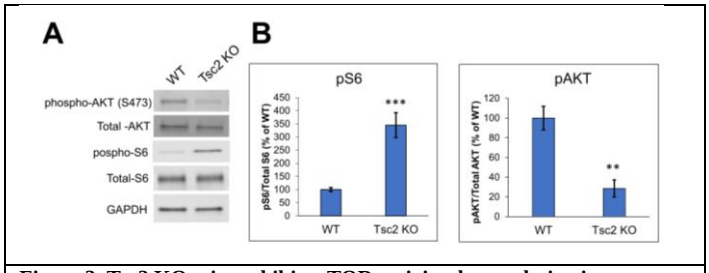

Figure 3. Tsc2 KO mice exhibit mTOR activity dysregulation in cortex. A) Western blot sample of cortex homogenate from P16 mice probed for phospho-S6 (pS6) as a readout of mTOR activity. B) pS6 is significantly increased while phospho-AKT is decreased in Tsc2 mouse cortex indicating hyperactivated mTOR.

Major Task 3. To identify synaptic connections and record electrophysiological properties of the neurons during early onset of epileptiform activity. Only recorded from 5 animals, and collected cell samples for RNA-seq. Have not processed the samples.

Specific Aim 2: To evaluate characteristics of the seizure-prone circuit and molecular signatures associated with mTOR inhibitor resistance in NEX-*Tsc2* KO mice.

Major Task1. Monitor epileptiform activity for emergence of seizures in KO mice with chronic RAD001 treatment. Previously it was shown that Tsc2 KO mice treated with a high, chronic dose of mTOR inhibitor RAD001 prolonged survival. It was not known if epileptiform or seizure activity was also rescued by RAD001 treatment. We first determined optimal dosage for thriving and survival since high, chronic dosage although prolonging survival also suppressed growth and resulted in early death. Based on literature review, an optimal RAD001 dosage of 6mg/kg every two days was determined to prolong survival while also allowing weight gain. Subsequently, it was possible to implant mice for EEG studies. Implanted mice were recorded continuously throughout treatment with RAD001. EEGs revealed that seizures and epileptiform activity is suppressed by RAD001 treatment but breakthrough seizures appear at approximately postnatal day 45. Furthermore, quantitative EEG analysis revealed that spectral power was not rescued by RAD001 treatment, indicating an ongoing circuit dysfunction that is resistant to mTOR inhibition. Treated, implanted and recorded from 35 mice (WT Veh, WT RAD, Tsc2 KO Veh, Tsc2 RAD).

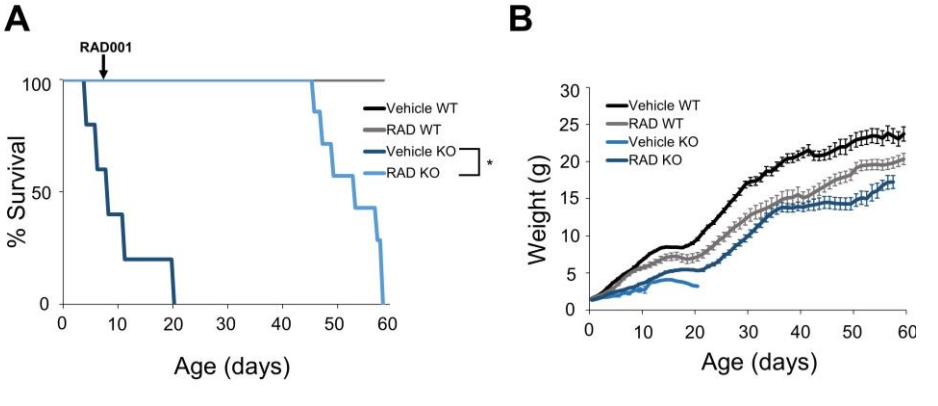

Figure 4. Tsc2 KO mice thrive after early postnatal treatment with the mTOR inhibitor, RAD001. Tsc2 KO mice treated chronically with RAD001 survive significantly longer than vehicle-treated KO mice and B) exhibit weight gain throughout treatment.

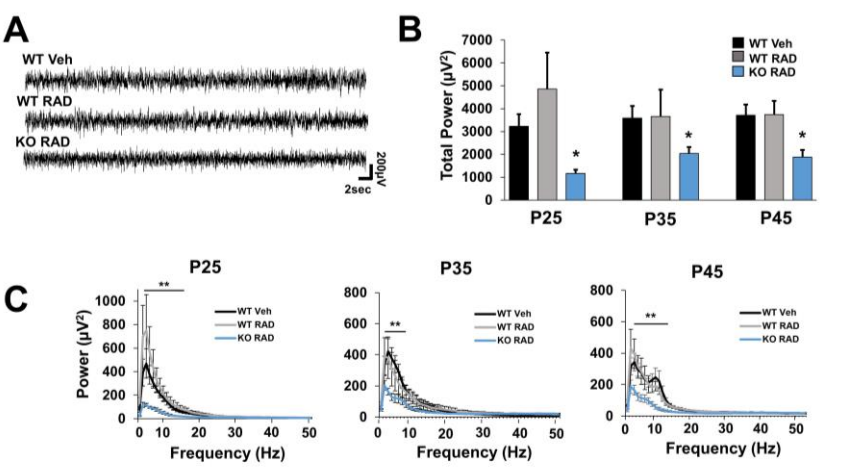

Figure 5. Tsc2 KO EEG power deficits are not rescued by treatment with RAD001. A) Sample EEG traces from WT and Tsc2 KO mice during vehicle or RAD001. B) Total and C) spectral EEG power remain significantly decreased relative to WT throughout treatment with the RAD001. Note: Vehicle treated KO mice die prematurely (see figure 4).

Major Task 2. Assessment of mTOR activity levels in KO and WT mice treated with RAD001. We wanted to determine if the breakthrough seizures correlate with impaired mTOR dysregulation during RAD001 treatment. We collected brain tissue from WT and Tsc2 KO mice at various time points during treatment with vehicle or RAD001. After starting RAD001 during early development it was revealed that pS6 levels are normalized from P25-P35 but is significantly elevated at P45. These results suggest a possible resistance to mTOR inhibitor treatment. These studies will inform subsequent microcircuit experiments aimed at determining pre- and post-drug resistance changes in gene expression. We have used 51 animals for tissue collection (3/group WT Veh, WT RAD, KO Veh, KO RAD from ages P8, P16, P25, P35, and P45). No tissue cannot be collected from KO Veh >P16 due to premature death.

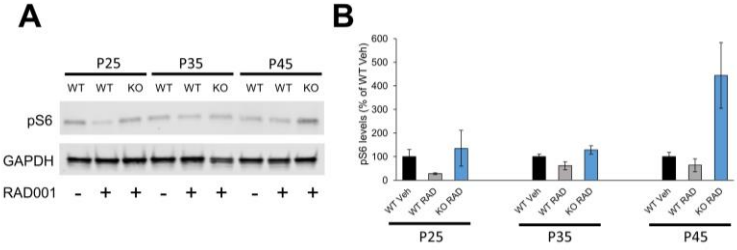

Figure 6. Treatment with RAD001 shows short-term suppression of mTOR hyper-activation in Tsc2 KO mice. A) Sample western blot of cortex homogenate from Tsc2 KO and WT treated with vehicle or RAD001 probed for pS6. B) Densitometry shows normalized levels of pS6 in Tsc2 KO mice at postnatal day 25 and 35, however, there is increased levels at postnatal day 45 relative to the WT groups.

Major Task 3. Determine the microcircuitry changes and cell type- specific transcriptomic signatures in pre- and post- development of RAD001-resistance in KO mice. Not yet initiated.

What opportunities for training and professional development has the project provide?
Dr. Luis Martinez has been learning biochemistry and electrophysiology techniques through this project and is also preparing a manuscript from the first year of work. In addition, we have a volunteer undergraduate neuroscience major,

How were the results disseminated to communities of interest?
Nothing to Report

What are you planning to do the next reporting period to accomplish the goals?
We will complete the immunohistochemistry of the tissue collected from both specific aims. As critical pre- and post-epilepsy timepoints in the molecular and EEG studies are completed from Major Tasks 1 and 3, we will carry out the experiments on Major Task 3 under both Specific aims: determine differentially expressed genes (DEGs) pre- and post-epilepsy during early development and determine DEGs pre- and post-seizure re-emergence during RAD001 treatment.

4. IMPACT

What was the impact on the development of the principal discipline of the project?
We found that RAD001 was able to suppress seizures in Tsc2 KO mice with conditional deletion of the Tsc2 gene in excitatory neurons. Breakthrough seizures appear during treatment and their re-emergence is associated with an increase in mTOR activity.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
The reemergence of the seizures during treatment with the mTOR inhibitor parallels what is often observed in TSC patients. These results provide an ideal model that allows the study of molecular mechanisms of mTOR inhibitor resistance which may ultimately have an impact on changing clinical practice and decision making.

5. CHANGES/PROBLEMS:

- ***Changes in approach and reasons for change***
  Nothing to Report

- ***Actual or anticipated problems or delays and actions or plan to resolve them***
  Nothing to Report

- ***Changes that had a significant impact on expenditures***
  Due to supply chain issues had impact on experiments. The experiments carried out by the Jiang's lab has been delayed. Need to have funds not spent year 1 transferred to years 2-3 for my lab – Dr. Martinez to create mice, EEG monitoring, and collect tissue for Jiang lab who will need funds for RNA-seq processing/personnel.

- ***Significant changes in user or care of human subjects, vertebrate animals, biohazards, and/or selected agents***
  Nothing to Report

- ***Significant changes in user or care of human subjects***
  Nothing to Report

- ***Significant changes in user or care of vertebrate animals, biohazards***
  Nothing to Report

- ***Significant changes in user or care of selected agents***
- Nothing to Report

6. PRODUCTS:

- **Publications, conferences papers, and presentations**

  - **Journal publications**
    Nothing to Report

  - **Bookss or othe non-periodical, one-time publications**
    Nothing to Report

  - **Other publications, conference papers and presentations**
    Nothing to Report

- **Website(s) or other Internet site(s)**
  Nothing to Report

- **Technologies or techniques**
  Nothing to Report

- **Invention, patent applications, and/or licenses**
  Nothing to Report

- **Other Products**
  Nothing to Report

7. PARTICIPANTS AND OTHER COLLABORATING ORGNIZATIONS

What individuals have worked on the projects?

1. Anne Anderson (PI) – no change
2. Xiaolong Jiang (Co-PI)- no change
   Luis Martinez (Postoctoral fellow) – no change

Name:	
Project Role:	
Researcher Identifier:	
Nearest person month worked:	
Contribution to Project:	
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Anderson (PI)
NEW ACTIVE SUPPORT
Assessing determinants of electrocardiographic alterations and identifying exacerbating factors in pediatric epilepsy
NIH/NINDS R21 NS119929-01A1 (PI: Yi-Chen Lai)
Goal is to study epilepsy duration on cardiac remodeling as a contributor of SUDEP
07/01/2021-12/31/2022
(3.59%) Role: Co-I
Program Official: Vicky R Whittemore Email: whittemorevr@mail.nih.gov Phone:
Grant Management Specialist: Donna M James Email: jamesd@ninds.nih.gov Phone:

No overlap

Electrophysiological Approaches to Understanding Functional Organization of Speech in the Brain
NIH/NINDC R01 DC018579 (PI: Liberty Hamilton)
The goal is studying electrophysiology activity during speech perception and production in the developing brain
12/01/2020-11/30/2025
(5%) Role: Co-I
Program Official: Renee Gonzales, Office of Sponsor Projects spaa@austin.austin.utexas.edu The University of Texas at Austin, Office of Sponsored Projects, 3925 West Braker Lane, Building 156 (WPR), Suite 3.340, Austin TX 78759. Phone:
No overlap

Jiang (Co-PI)
CLOSED
Reverse engineering neocortical intelligence
Machine Intelligence from Cortical Networks (MICrONS) IARPA #D16PC0003 (PI: Tolias, A., Pitkow, Z., etc.)
We will combine three-photon imaging (3PI) and in vivo 3D Random Access Multi-Photon Imaging (3DRAMP) methods to develop a novel 2P/3P imaging system and record from all 100,000 neurons in 1 mm3 of cortex in vivo, across all six neocortical layers, and three hierarchically organized visual areas in a behaving mouse. We will further use a multi-beam Scanning Electron Microscope (SEM) to reconstruct the connectome of this cortical tissue, including reciprocal connections between the three cortical areas. Thus, with our combined expertise, we will be able to simultaneously monitor activity across all cortical layers during behavior, and thereby measure all of the transformations within the cortical column
01/15/16-01/14/21
(1%) Role: Co-Investigator

Contact: David A. Markowitz, Ph.D. david.markowitz@iarpa.gov
No overlap

What other organizations were involved as partners?
Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Not applicable

9. APPENDICES

Not applicable