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14. ABSTRACT
The goal is to identify ryanodine receptor type 1 (RYR1) gene mutations associated with enhanced susceptibility to Exertional Heat Stroke (EHS), Exertional Rhabdomyolysis (ER), and Malignant Hyperthermia (MH) by enrolling patients diagnosed with these conditions and performing genetic screening. During the project period, 37 patients were enrolled: 27 index cases and 10 family members from 2 of the index cases. Of the 27 index cases, 5 patients have 4 known MH-causative RYR1 gene mutations (Arg163Cys, Gly2434Arg, Arg2454Cys and Arg2163His), 2 have known MH-associated RYR1 variants, 2 have novel RYR1 variants, 3 have no RYR1 mutations or variants, and 16 are still in progress (one patient had a known MH-causative mutation and a novel variant). Of the 10 family members screened from 2 index cases, 5 family members were found to share the same known MH-causative RYR1 gene mutation. Of the common mutations, the Arg2454Cys was identified in an African American with a positive CHCT and a history of ER. Identification of this mutation in a patient with ER strengthens the link between MHS and ER. A new variant, Gly4820Arg, was identified in a family with a history of an awake MH-like death. The 3 ER CHCT positive patients that were negative for the RYR1 screen will be further analyzed for CACNA1S and CASQ1 gene mutations. In addition, Task 2 and milestones 3 and 4 have been accomplished. AICAR is effective in preventing ER in the heterozygous MH mice but not in the homozygous (and more sensitive) MHS pigs. We are investigating the reasons for this discrepancy.

15. SUBJECT TERMS
Exertional Heat Stroke, Exertional Rhabdomyolysis, Malignant Hyperthermia , genetic analysis of type 1 Ryanodine Receptor (RYR1) and 5-Aminoimidazole-4-carboxamide ribotide (AICAR).

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Introduction: Exertional and/or environmental heat stroke (ES) and exertional rha bdomyolysis (ER) has been reported in patients with diagnosis of Malignant Hyperthermia (MH). MH is a life-threatening pharmacogenetic disorder caused b y mutations in the ryan odine receptor type 1 gene (RYR1) encoding skeletal mu scle calcium release ch annel. Our goal is to id entify RYR1 mutations associated with enhanced suscep tibility to EHS/ER/MH by enrolling subjects diagnosed with these conditions and performing genetic screen ing. We also proposed to evaluate the ability of AICAR to prevent the MH response in MHS mice and pigs.

Task 1: Screen for RyR1 mutations					
1a: Sequencing	**USUHS**	**45 human samples**	**XXXX**	**XXXX**	**XXXX**
Milestone 1: Show that RyR1 mutations underlie cases of enhanced susceptibility to heat stroke			**__XX**	**XX__**	
Milestone 2 Publish findings					**__XX**

Body: During the project period, 37 patients (out of 45 proposed) with a history of EHS/ER or MH were e nrolled in this study. The RYR1 gene was screened in t hese individuals. RYR1 mutations and variants were identified in 13 of the 37 enrolled patients (see Table 1 attached). Four well known disea se causative MH mutat ions (Arg163Cys, Gly2434Arg, Arg2454Cys an d Arg2163His), two previously publish ed MH-associated mutations and tw o novel variants were identified in the RYR1 g ene. Of the four common mutations, the Arg2454Cys was id entified in an African American patient with a positiv e CHCT (validated diagnostic t est for MH susceptibility) and a history of ER. Since the Arg2454Cys mutation is characterized as causative for MH, identification of this mutation in a subject with ER further strengthens a link between MH and ER. A new variant, Gly4820Arg, was found in a family with a history of a dea th due to an awake MH-like event.

To date, 37 patients dia gnosed with MHS, ER and/or EHS and their family members have been enrolled (Table 1). Of th ese, 27 are index cases and 10 are first degree relatives of two of the index cases, BU-05 and BU-12 (Fig. 1). Enrollment of family members is important for studying the genotype and phen otype relationships, and to understand the pa thogenic significance of familial variants.

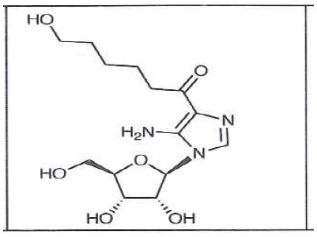
RYR1 gene mutations and variants have been found in 8 index cases, which account for 30% of the index cases. One patient, who died of an awake MH-like episode but was known to be MH susceptible, had a Ph e41Ser novel RYR1 variant and a Gly2434 Arg known MH-causative mutation. Of these two variants, t he Gly2434Arg is one of the most common pathogenic mutations in the RYR1 gene. The presence of the second variant may have contributed to the fatal outcome in th is case; however the fun ctional significance of the second varia nt requires further studies. Another novel variant, Gly4820Arg, was fou nd in the fat her of a child who had the same variant and died of an awake MH-like episo de. This fa ther was subsequently diagnosed MH susceptible by positive CHCT, and was further diagnosed with central core disease based on his muscle histopatholog y. Interestingly, another mutation at the same position, Gly4820Trp, was reported in association with MH susceptibility (Robinson et al., 2006).

Three patients who presented with ER, and who were dia gnosed MH susceptible by positive CHCT, did not have any RYR1 vari ants after complete scr eening of the gene. These patients will be further analyzed for CACNA1 S and CASQ1 gene mutations. The RYR1 gene screening continues in 16 other patients.

Task 2. Determine if AICAR's ability to rescue MH mice is due to effects on RyR1 or AMPK (animal protocol approved)					
2a. Indirect calorimetry to assess acute effects of AICAR, A769662, and derivatives for effects on VO2 in each mouse. Mice: WT, MH, AMPKαDN, AMPKγCA, MH/ AMPKαDN, and MH/ AMPKγCA	**BCM**	**8-10 of each line (6) per year: 60 (total for 3 years is 180)**	**XXXX**	**XXXX**	**XXXX**
2b. Determine effects of AICAR on AMPK and RyR1 phosphorylation	**BCM**	**Same mice as in 2a, 2c, 3a, 3b**	**XXXX**	**XXXX**	**XXXX**
2c. Bilayer, Binding, Calcium Imaging 6-8 week old mice are sacrificed and studies are performed with AICAR or A769662 added in vitro.	**BCM**	**8-10 of each line/yr 60/year total for 3 years is 180**	**XXXX**	**XXXX**	**XXXX**
Milestone 3: Demonstration of AICAR's ability to regulate RyR1 actlvity	**BCM**		**__XX**	**XXXX**	**XXXX**
Milestone 4: Publication of these data			**__XX**	**__XX**	**__XX**

Task 2 has been completed and the findings (milestone 4) published in 2012.

Lanner, J., Georgiou, D.K., Dagnino-Acosta, A., Ainbinder, A., Cheng, Q., Joshi, A., Chen, Z., Yarotskyy, V., Oakes, J., Lee, C.S., Monroe, T., Santillan, A., Dong, K., Goodyear, L., Ismailov, I., Rodney, G.G., Dirksen, R., an d Hamilton, S. AICAR Prevents Heat Induced Malign ant Hyperthermia in RyR1 Mutant Mice Independe nt of AMPK Activation, Nature Medicine,18: 244 – 251, 2012 PMID: 22231556 [PubMed - as supplied by publisher.



However we found that AICAR requi res extremely hig h concentrations and hen ce we established a collaboration with Prof. Gennaro Piccialli to sy nthesize and test AICAR derivative s to find a more potent analog. We have a very good candidate (structure shown to the right) that is much more potent in [^{3}H]ryanodine binding assays than AICAR. We are prepa ring to test it in the MH mice and then in the MHS pigs which, as discussed b elow, are not r escued by AICAR, most likely due to our i nability to achieve an effective concentration.

Task 3. Assess effects of chronic treatment with AICAR on MH response and endurance					
3a. Assess effects of chronic treatment with AICAR and A769662 on endurance, force and fatigue. Mice are treated for 6 weeks while on monitored running wheels. At the end of 6 weeks the mice are sacrificed and force-frequency and fatigue studies are performed.	**BCM**	**8-10 of each line/yr 60/year total for 3 years is 180**	**XXXX**	**XXXX**	**XXXX**
3b. Assess effect of chronic treatment with AICAR and/or A769662 on heat response by indirect calorimetry (mice are treated for 6	**BCM**	**8-10 of each line/yr 60/year total**	**XXXX**	**XXXX**	**XXXX**

weeks while on monitored exercise wheels and then subject to indirect calorimetry)		**for 3 years is 180**			
Milestone 5: Determination of whether AICAR affect on endurance is due to effects of RyR1, AMPK or both.	**BCM**		**XXXX**	**XXXX**	**XXXX**
Milestone 6 Publication of Milestone 5					**__XX**

These studies have been initiated but have not yet been completed. The mice will be treated with AICAR (600mg/kg) once a week for 6 weeks and we will assess the ER response, endurance and muscle performance

Task 4. Assess the ability of AICAR to prevent MH repsonse in MHS susceptible pigs					
4a. Modify animal protocol	**USUHS**		**X____**	**____**	
4b. Test AICAR in MHS pigs and controls	**USUHS**	**6 normal and 6 MHS pigs**	**__XX**	**XX**	
4c: Therapeutic use of AICAR vs Dantrolene	**USUHS**	**16 MHS pigs**	**____**	**_-XX**	**XX**
Milestone 7: Establish ability of AICAR to rescue MHS pigs				**__XX**	**__XX**
Milestone 8: Publish findings			**____**		**__XX**

We found that AICAR, when administered as a pretreatment or as a rescue drug, was not effective as a treatment for heat-induced MH in the MH susceptible swine. This finding appeared to be in conflict with data obtained in the MH susceptible transgenic YS mouse model. We hypothesized that in the pig model AICAR (600mg/kg IV) may not achieve an effective concentration in the swine skeletal muscle. To test this possibility, we examined the effect of AICAR on isolated muscle preparations from the MHS pigs.

Six MHS swine were anesthetized as previously described with a total intravenous propofol infusion and euthanized at the end of the already approved in vivo protocol. While receiving propofol general anesthesia during the approved portion of the protocol, a 2 inch incision was made on the ventral surface of the swine's lower limb. The skin was shaved, cleaned and prepped with alcohol before incision. Employing sterile technique, a number 15 scalpel blade was used to incise the skin, while surgical scissors and forceps were used to expose the underlying muscle. The muscle was dissected and excised using scalpel, surgical scissors and forceps, and placed on tension in two specialized clamps. The muscle specimen was measured 1.0 inch by 0.5 inch, and it was immediately placed in a Krebs-Ringer solution. The muscle was taken to another laboratory for analysis in the *in vitro* contracture test (Table 2), using the standard procedures for the performance of the caffeine halothane contracture test as described by the North American Malignant Hyperthermia Group (Larach 1989). The results are shown in Table 2.

AICAR at 1mM and 10mM did not alter basal tension of muscle strips, (P = 0.96 and 0.86) when compared to baseline tension of untreated control strips. Similarly AICAR (1 and 10mM) did not significantly alter halothane- or caffeine-induced contractures.

The results (Table 2) from the in vitro studies from MHS swine indicate that AICAR had no effect at both low and high concentrations on muscle tension development in basal conditions, nor did AICAR influence responses induced by caffeine and halothane challenges. This is consistent

with the previous report that AICAR is ineffective when administered intravenously in the MHS pig model.

Key accomplishments:

- Further support that RyR1 mutations underlie ER/EHS
- Publication of AICAR mechanism of action to prevent EHS
- Identification of AICAR derivative with higher potency
- Case report publication of awake MH

Reportable Outcomes:

- 37 patients diagnosed with MHS, ER and/or EHS and their family members have been enrolled (Table 1). Of these, 27 are index cases and 10 are first degree relatives of two of the index cases.
- RYR1 gene mutations and variants have been found in 8 index cases which account for 30% of the index cases.
- One patient who died of an awake MH-like episode had two RYR1 gene variants, Phe41Ser and Gly2434Arg. Of these two variants, the Gly2434Arg is one of the most common pathogenic mutations in the RYR1 gene.
- Two novel variants, Gly4820Trp and Phe41Ser were found in two independent cases.
- Published: Lanner, J., Georgiou, D.K., Dagnino-Acosta, A., Ainbinder, A., Cheng, Q., Joshi, A., Chen, Z., Yarotskyy, V., Oakes, J., Lee, C.S., Monroe, T., Santillan, A., Dong, K., Goodyear, L., Ismailov, I., Rodney, G.G., Dirksen, R., and Hamilton, S. AICAR Prevents Heat Induced Malignant Hyperthermia in RyR1 Mutant Mice Independent of AMPK Activation, Nature Medicine,18: 244 – 251, 2012 PMID: 22231556 [PubMed - as supplied by publisher.
- We discovered an AICAR derivative that is more potent that AICAR
- AICAR at 1mM and 10mM did not alter basal tension of pig muscle strips, (P = 0.96 and 0.86) when compared to baseline tension of untreated control strips.
- Similarly, AICAR (1 and 10mM) did not significantly alter halothane- or caffeine-induced contractures.
- AICAR is ineffective when administered intravenously in the MHS pig model, but the more potent derivative will now be tested
- Manuscript in print in Anesthesia and Analgesia. “Death in the Emergency Department: An unrecognized awake MH-like reaction in a six year old”.

Conclusions:

- RYR1 mutations underlie a significant number of cases of exercise induced rhabdomyolysis and environmental heat stroke.
- AICAR prevents EHS in mice heterozygous for RYR1 mutation associated with MH in humans but not in pigs that are homozygous for a different mutation.
- Derivatives of AICAR may be more potent for preventing EHS/ER than AICAR

References:

Robinson R, Carpenter D, Shaw M-A, Halsall J, Hopkins P (2006) Mutations in *RYR1* in malignant hyperthermia and central core disease. *Hum Mutat* 27:977-89.

Appendices:

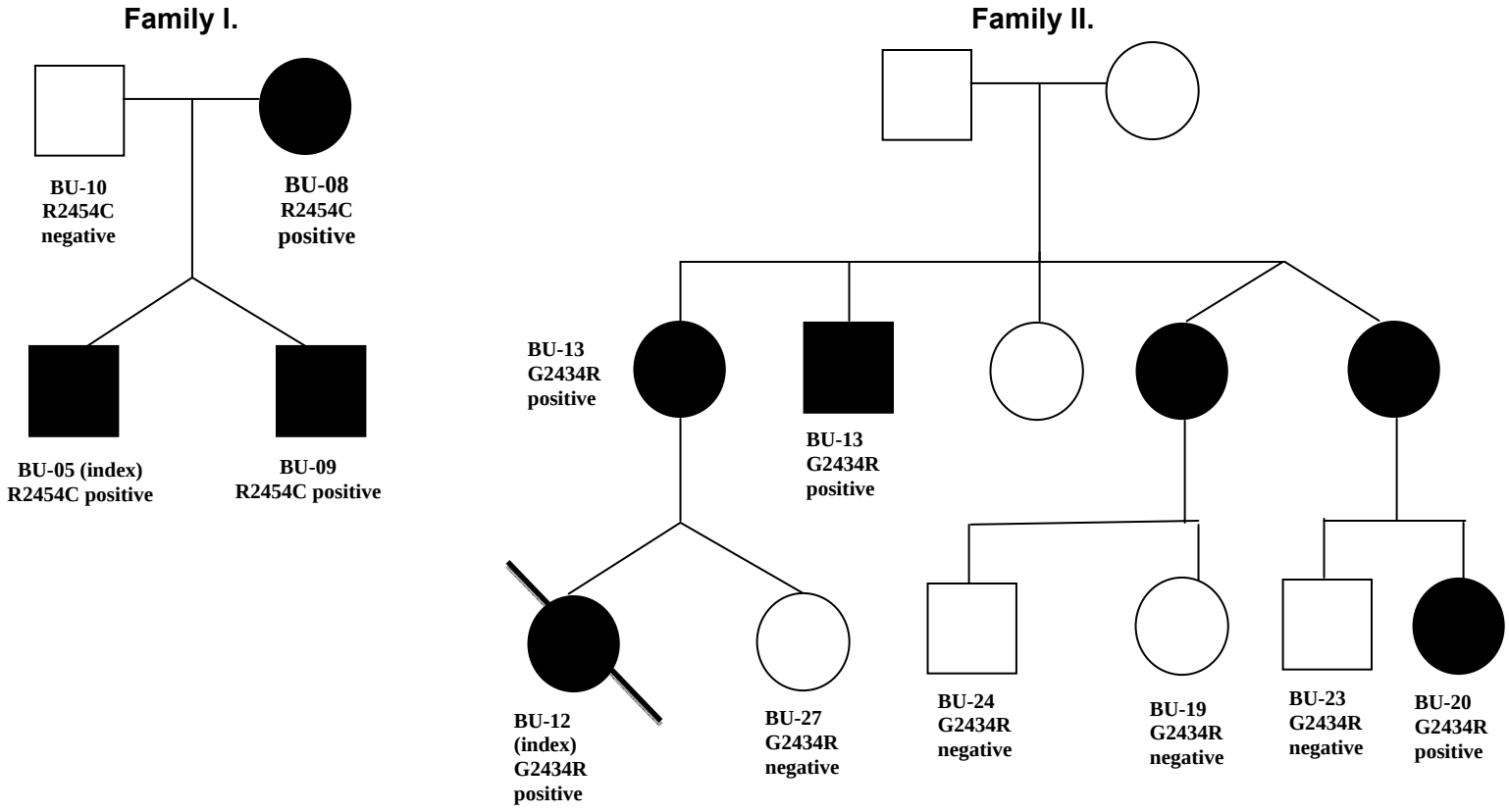
Table. 1 Results of RYR1 gene screening in enrolled subjects.

Report ID	Clinical history	CHCT Results	Index or Family members	Results of genetic screening
BU-01	Exercise induced rhabdomyolysis, heat related death in a family member	positive	index	**Gly4820Arg**
BU-02	Muscle pain with exercise post MH event	positive	index	**Arg163Cys**
BU-03	Exercise induced rhabdomyolysis, MHS	positive	index	In progress
BU-04	Exercise and heat intolerance, muscle cramping and MH	positive	index	**Asp3986Glu**
BU-05	Repeated Exercise induced rhabdomyolysis	positive	index	**Arg2454Cys**
BU-06	Repeated Exercise induced rhabdomyolysis	n/a	index	**Gly2434Arg**
BU-07	Death due to MH like event	n/a	index	In progress
BU-08	No known clinical history	positive	Mother of BU-05	**Arg2454Cys**
BU-09	Cardiac arrest during surgery	n/a	Twin brother of BU-05	**Arg2454Cys**
BU-10	No known clinical history	n/a	Father of BU-05	Negative for **Arg2454Cys** familial mutation
BU-11	Traumatic MH Episode	n/a	index	**Arg2163His**
BU-12	Death due to MH and Exercise/heat intolerance	n/a	index	**Phe41Ser & Gly2434Arg**
BU-13	MH and heat related death in family member	n/a	Mother of BU-12	**Gly2434Arg**
BU-14	Exercise and heat intolerance	Positive	index	In progress

BU-15	Exertional Rhabdomyolysis	Positive	index	**Val4842Met**
BU-16	Exercise and heat intolerance	Negative	Index	In progress
BU-17	Family history of MH	n/a	Index	In progress
BU-18	Exercise/heat related intolerance, heat stroke and mother had suspected MH episode	n/a	index	In progress
BU-19	MH and heat related death in family member	n/a	1st Cousin of BU-12	Negative for **Gly2434Arg** familial mutation
BU-20	MH and heat related death in family member	n/a	1st Cousin of BU-12	**Gly2434Arg**
BU-21	Exertional Rhabdomyolysis	Positive	index	In progress
BU-22	MH and heat related death in family member	N/A	Uncle of BU-12	**Gly2434Arg**
BU-23	MH and heat related death in family member	N/A	1st Cousin of BU-12	Negative for **Gly2434Arg** familial mutation
BU-24	MH and heat related death in family member	N/A	1st Cousin of BU-12	Negative for **Gly2434Arg** familial mutation
BU-25	Heat and Exercise intolerance	N/A	index	In progress
BU-26	Exertional Rhabdomyolysis/ Heat & Exercise Intolerance	Negative	index	In progress
BU-27	MH and heat related death in family member	N/A	Twin sister to BU-12	Negative for **Gly2434Arg** familial mutation
BU-28	Exertional Rhabdomyolysis	Positive	index	Genetic screen completed, negative for RYR1 mutation
BU-29	Exertional Rhabdomyolysis	Positive	index	In progress
BU-30	Exertional Rhabdomyolysis	Positive	index	Genetic screen completed, negative for RYR1 mutation
BU-31	Exertional Rhabdomyolysis	Positive	index	Genetic screen completed, negative for RYR1 mutation
BU-32	Exertional Rhabdomyolysis	Negative	index	In progress
BU-33	Exertional Rhabdomyolysis	Negative	index	In progress

BU-34	Exertional Rhabdomyolysis	Negative	index	In progress
BU-35	Exertional Rhabdomyolysis	Negative	index	In progress
BU-36	Exertional Rhabdomyolysis	Negative	index	In progress
BU-37	Exertional Rhabdomyolysis	Negative	index	In progress

Pedigree Analysis (Fig.1)



Pedigrees of two unrelated MHS and Exertional Rhabdomyolysis families. Filled symbols denote individuals with familial mutations and or carriers of familial mutations. Filled symbol with bisecting line indicates individual that died from MH/Exercise and heat intolerance (BU-12). R or C denote Arginine or Cysteine at amino acid 2454 sequence position; G or R denote Glycine or Arginine at amino acid 2434 sequence position of the RYR1gene respectively. Unlabeled symbols denote individuals that were not screened under this protocol.

Table 2. Effect of AICAR on basal tension and 3% halothane-, 2mM caffeine-induced contractures on muscle strips from MHS swine.

Treatment	Twitches (g)	Baseline (0 min) Tension (g)	Tension 30 min Post	Tension 90 min Post	3% Halothane-Induced Contracture (g)	2 mM Caffeine-Induced Contracture (g)
Control	3.25 ± 0.41 n = 36	2.18 ± 0.08 n = 36	1.81 ± 0.11 n = 36	1.74 ± 0.13 n= 18	3.26 ± 0.36 n= 18	1.16 ± 0.13 n = 14
1mM AICAR	3.55 ± 0.68 n = 18	2.11 ± 0.11 n = 18	1.68 ± 0.09 n = 18	1.72 ± 0.14 n = 9	2.99 ± 0.46 n = 9	1.37 ± 0.28 n = 7
10mM AICAR	4.6 ± 0.95 n= 18	1.82 ± 0.12 n = 18	1.30 ± 0.13 n = 18	1.33 ± 0.15 n = 9	2.93 ± 0.32 n = 9	1.43 ± 0.18 n = 7

Data were analyzed using two way ANOVA and are presented as Mean ± SEM, n = number of muscle strips tested. Muscle strips from MHS swine were mounted in an organ bath and after initial equilibration period (10-15 min) muscle strips were treated with distilled water with and without (control) AICAR (1 and 10mM). Basal tension of strips was recorded continuously up to 90mins. After 90mins period, muscle strips were treated with either 3% halothane or 2mM caffeine.

QUARTERLY REPORT FORMAT

1. Award No. W81XWH-10-2-0117 2. Report Date 10/16/2012

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4. PI: Susan Hamilton & Sheila Muldoon/John Capacchione
5. Telephone No. 713-798-5704

6. Institution: Baylor College of Medicine & Uniformed Services University of the Health Sciences (USUHS)

7. Project Title: Novel Interventions for Heat/Exercise Induced Sudden Death and Fatigue

8. Current staff, with percent effort of each on project.

Baylor

Susan Hamilton, Ph.D. 10 %
Dimitra Georgiou, Ph.D. 100 %
Keke Dong 20 %

USUHS

Sheila Muldoon, M.D 5%
John Capacchione, M.D 5%
Francis O'Connor, M.D. 5%
Nyamkhishig Sambuughin, Ph.D. 5%
Rolf Bunger, M.D. 5%
Tarina Wallace, MS 50%

9. Award expenditures to date (as applicable):

	This Qtr/Cumulative		**This Qtr/Cumulative**
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Fringe Benefits	_____	Equipment	_____/_____
Supplies	_____	Other	_____

	This Qtr/Cumulative
Subtotal	_____
Indirect Costs	_____
Fee	_____/_____
Total	_____

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11. Use additional page(s), as necessary, to describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this assistance agreement.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.