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14. ABSTRACT

Diabetics have more heart disease than their non-diabetic counterparts, even though drugs like statins are equally effective in both groups at lowering the blood levels of harmful cholesterol. We have identified an enzyme called PRMT2, which regulates the abundance of a cellular cholesterol transporter that helps to prevent cells from accumulating in arteries and forming a plaque. We have shown that the level of PRMT2, while high in healthy cells, is very low in cells from diabetics when blood sugar levels are elevated. Because PRTM2 isn't around in cells under diabetic conditions, we predict that more cells accumulate in the artery, thus allowing the plaque to grow and exacerbating heart disease in diabetics. To test this, we will determine what happens to the growth of a plaque in an artery when we eliminate PRMT2 with and without diabetes in mouse models of heart disease. We expect that plaques will grow larger in the absence of PRMT2. To better understand how PRMT2 suppresses plaque growth, we will also identify proteins that are modified by PRMT2 in cells from the plaque and determine if these proteins participate in plaque formation. Given that we also don't understand why PRMT2 levels decrease in diabetes, we will identify cellular proteins that regulate PRMT2 levels. That knowledge might enable us to develop ways to restore the normal level of PRMT2 in diabetes, and prevent the cells from contributing to plaque formation, and reduce heart attacks.

15. SUBJECT TERMS
atherosclerosis regression, diabetes, PRMT2, asymmetric arginine demethylation

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1. INTRODUCTION:

High plasma cholesterol and diabetes are major risk factors for atherosclerosis. People with diabetes are 2-4 times more likely to suffer from coronary heart disease (CHD). Given that 1 in 4 veterans who receive care from VA hospitals has diabetes testifies to the importance of this problem for military personnel. In human diabetics, treatment with cholesterol-lowering drugs such as statins fails to fully reduce the risk for atherosclerosis. Understanding the factors that are altered as a result of impaired glucose homeostasis is a significant area of cardiovascular research that has important ramifications for the health and well-being of the military workforce. Established coronary lesions of adulthood begin in childhood. Over time macrophages accumulate cholesterol, which promotes their differentiation into foam cells that become trapped in the artery, contributing to the growth of atherosclerotic plaques. Owing to a diminished capacity of cholesterol-engorged macrophages to migrate, they accumulate and fail to resolve inflammation, which leads to the plaque becoming unstable and rupturing, resulting in heart attacks and stroke. Accordingly, an important clinical goal to reduce CHD risk is to promote the regression of atherosclerosis by eliciting macrophage cholesterol efflux and macrophage migration from plaques.

Work from our labs has shown that regression of atherosclerosis is mediated in part by the Liver X Receptor (LXR) family of nuclear receptors through the induction of genes involved in cholesterol efflux. Given that we also found that regression of atherosclerosis is impaired in the context of diabetes, we proposed that changes in glucose levels modulate LXR-dependent gene expression and reduce the expression of LXR target genes like the cholesterol transporter protein *ABCA1*, and that this molecular mechanism might account for the increased rate of atherosclerosis in diabetics. We showed that LXR-mediated *ABCA1* expression and ABCA1-dependent cholesterol efflux is compromised in macrophages exposed to high, compared to normal, levels of blood glucose. Moreover, we identified the protein arginine methyltransferase 2 (PRMT2), cellular levels of which are reduced in high versus normal glucose, as a factor that mediates this effect. Macrophages devoid of PRMT2 showed reduced LXR-dependent induction of *ABCA1* and reduced ABCA1-mediated cholesterol efflux, thus mimicking the effect of high glucose. Expression of *PRMT2* was lower in monocytes from diabetic mice. Our studies revealed PRMT2 as a glucose-sensitive factor that plays a role in the induction of *ABCA1* by LXR and affects cholesterol efflux. Thus, PRMT2 might be critical in mediating the increased incidence of atherosclerosis in diabetics. The goal of this proposal is to understand how PRMT2 deficiency promotes atherosclerosis during diabetes.

2. KEYWORDS:

KEYWORDS: PRMT2, atherosclerosis regression, diabetes, macrophages, asymmetric dimethyl arginine, regulation of gene expression

3. ACCOMPLISHMENTS:

- What were the major goals and objectives of the project?

The major goals of the project are:

1) determine the role PRMT2 plays in the impaired regression of atherosclerosis in diabetes
2) determine the substrates of PRMT2 in macrophages in normal and high glucose
3) determine the molecular regulation of PRMT2

- What was accomplished under these goals? Major activities for this reporting period:

Specific Aim 1: To determine the role PRMT2 plays in the impaired regression of atherosclerosis in diabetes

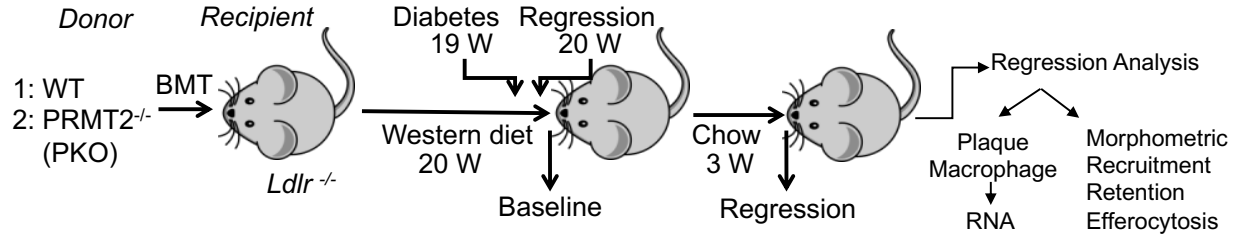
Major Task 1: Bone Marrow transplant from *PMRT2*$^{-/-}$ into *LDLR*$^{-/-}$ mice **(completed)**

Subtask 1: Generate *PMRT2*$^{-/-}$ and WT mice for bone marrow transplant into *LDLR*$^{-/-}$ mice

Subtask 2: Perform bone marrow transplants of *PRMT2* $^{-/-}$ into *LDLR*$^{-/-}$ mice and enter mice into study protocols

We have established a colony of PRMT2$^{-/-}$ mice to determine how PRMT2 affects atherosclerosis regression in diabetes. We used these mice to perform bone marrow transplants into atherosclerosis-prone LDLR$^{-/-}$ mice and examined the effect on atherosclerosis regression with and without diabetes. We used a bone marrow transplant approach from wild type (WT) and PRMT2$^{-/-}$ mice, since this has the potential to uncover the importance of PRMT2 activities within myeloid-derived immune cells, while avoiding confounding effects of PRMT2 expression in other organs. This was crucial given that mice with a whole body knock out PRMT2, while viable and fertile, were defective in leptin signaling by virtue of PRMT2's effect on STAT3 methylation and signaling in the hypothalamus (1).

The bone marrow transplant/atherosclerosis regression studies were performed using the following scheme (Fig.1). After bone marrow transplant, mice were allowed to recover for 4 weeks, then placed on a high fat, high cholesterol "western diet" for 20 weeks to promote the formation of advanced atherosclerotic plaques. At 19 weeks, half the mice were made diabetic using STZ, and the other half were not, and injected with citrate buffer as a control. To induce regression, mice were treated with an anti-sense oligonucleotide (ASO) against the *APOB* gene to reduce LDL cholesterol levels and promote regression. Mice were placed on chow diet for 3 weeks, plaques harvested and plaque area measured.



Major Task 2: Analysis of aortic arches from regression cohort -/+ PMRT2, -/+ diabetes

Subtask 1: Sac mice, collect serum, harvest, fix and embed aortic tissues **(in progress)**

Figure 1. Experimental design for the regression of atherosclerosis studies. *LDLR-/-* mice were irradiated to kill the endogenous bone marrow cells, and bone marrow transplants into them were performed from *PRMT2*$^{-/-}$ or littermate wild type controls. Mice were allowed to recover for 4 weeks and placed on a western diet for 20 weeks. A group of animals was sacrificed after 20 weeks of western diet feeding and used as the baseline group. Two other groups of animals received citrate buffer or STZ to induce diabetes at 19 weeks. At 20 weeks, to lower LDL cholesterol, regression and regression/STZ groups received *APOB* anti-sense oligonucleotide (ASO) and were switched to a chow diet. Animals were sacrificed 3 weeks later and the indicated parameters analyzed.

The *PRMT2*$^{-/-}$ bone marrow recipients had similar body weight, cholesterol and glucose levels compared to their WT bone marrow recipient counterparts (Fig. 2A-C; see 19 week columns). As expected, the glucose levels were dramatically higher in STZ-treated mice than in control mice (Fig. 2C; see green and black bars, Reg 3 weeks). Similar reductions in cholesterol were observed in normal and diabetic mice upon reversal of hyperlipidemia (Fig. 2B; see Reg 3 weeks).

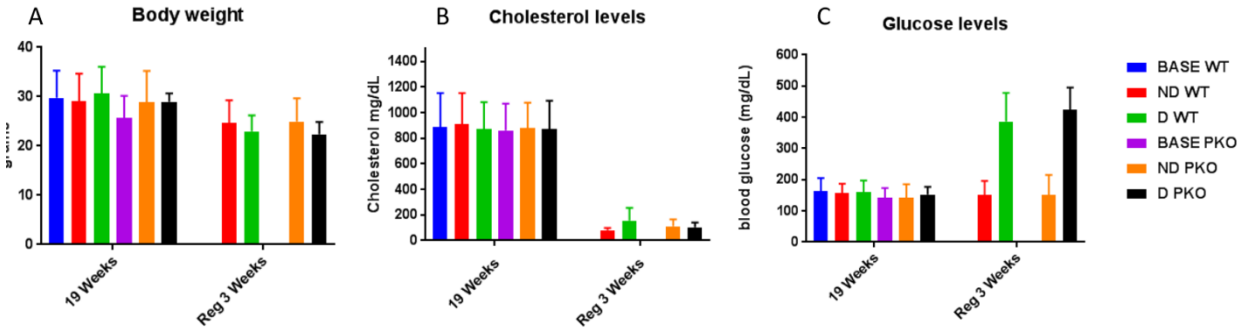


Figure 2. Parameters of the PMRT2$^{-/-}$ and WT bone marrow transplant mice. Mice weights, total cholesterol and blood glucose levels were determined in wild type and *PRMT2*$^{-/-}$ cohorts. Base= baseline; ND= non-diabetic; D= diabetic; WT= wild type; PKO= PRMT2 knock out (*PRMT2*$^{-/-}$ bone marrow). The bars above "19 weeks" are the measurements taken after 19 weeks on western diet (progression phase). The bars above the "Reg 3 weeks" are measurements taken at the end of the 3-week regression period.

To determine the effect of PRMT2 on atherosclerosis regression, we measured plaque area and total macrophage content by quantifying the percent of the macrophage marker CD68 (Fig. 3). We have reported that hyperglycemia impaired atherosclerosis regression (2). The preliminary results show the following: *PRMT2*$^{-/-}$ baseline mice had higher macrophage contents in their plaques compared to WT mice. After 3 weeks of reduction in plasma cholesterol levels, we observed reduced macrophage content in the normoglycemic mice expressing WT and *PRMT2*$^{-/-}$ indicative of atherosclerosis regression. As expected, regression in WT mice under diabetic conditions mice was impaired. By contrast, *PRMT2*$^{-/-}$ mice did not alter plaque macrophage content under diabetic versus non-diabetic conditions: in other words, regression was independent of diabetes in *PRMT2*$^{-/-}$ mice (Fig. 3). This is consistent with PRMT2 expression being low under hyperglycemia. The remaining mice in each group are in the process of being similarly analyzed. For all of the mice, RNA samples have been isolated from plaque macrophages using laser capture microdissection and submitted for RNA-seq to infer the factors and pathways regulated by PRMT2 based on bioinformatic analysis.

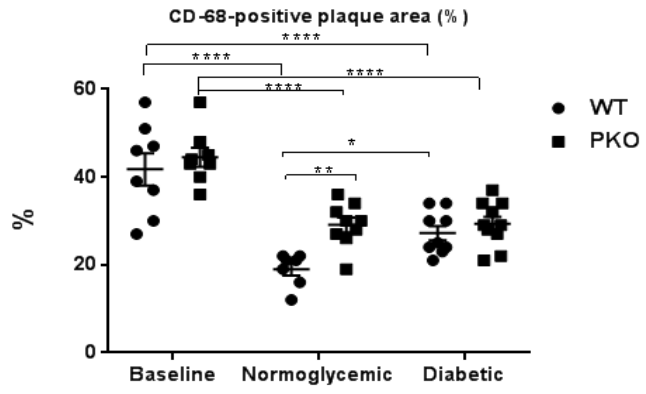


Figure 3. PRMT2 expression affects plaque macrophage content in diabetic mice. Aortic roots from baseline and regression groups from WT and PRMT2-/- (PKO) without or with diabetes were sectioned, fixed, and stained for CD68. The percent area occupied by CD68+ cells of the plaque area was quantified using ImagePro Plus Software. Data (mean ± SEM) (n≥8) were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. $P < 0.05$ values were considered to be significant. *P < 0.05, **P < 0.005, ***P < 0.0005.

Specific Aim 2: To determine the substrates of PRMT2 in macrophages in normal and high glucose that affects LXR transcriptional activity.

Major Task 1: Conduct studies to assess the PRMT2 substrate capture by immunoprecipitation (IP) using asymmetric arginine dimethylation antibody. **(in progress)**

We have made progress in the potential mechanism for PRMT2's effects. We have identified Histone H3 arginine 8 asymmetric dimethylation (H3R8Me2a) as a substrate for PRMT2

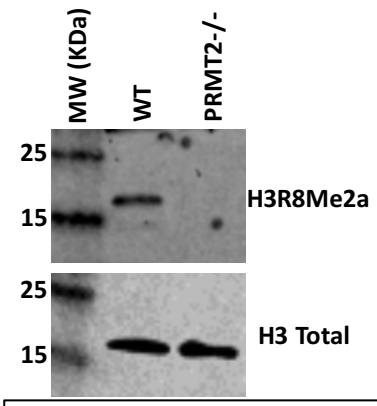


Figure 4. Histone H3 arginine 8 is a PRMT2 substrate. Extracts from BMDMs from wild type (WT) and *Prmt2* $^{-/-}$ mice cultured under normal glucose were immunoblotted with an antibody specific for Histone H3 asymmetric dimethyl arginine 8 (H3K8Me2a) and for total Histone H3 (H3 Total).

(Fig. 4). This is consistent with PRMT2's previously reported *in vitro* H3R8me2a methylation activity using either peptides or recombinant histones as substrates (3). It has also been shown by ChIP-seq in cell culture models of glioblastoma that H3R8Me2a is important for the maintenance of active promoters and enhancers (4). The H3R8Me2a modification has also been reported to promote an association with Tudor-domain containing proteins such as Spindlin-1 to regulate gene transcription (5). Tudor domain proteins function as molecular adaptors, binding methylated arginine on substrates to promote the assembly of macromolecular complexes including those involved in chromatin modifications.

We have also characterized PRMT2 substrates in an unbiased manner by proteomic analyses of macrophages cultured under normal glucose concentrations from WT and *PRMT2*$^{-/-}$ BMDMs (Fig. 5). We used affinity purification with an antibody specific to asymmetric arginine dimethylation to identify PRMT2 substrates. We were able to see an enrichment of 120 asymmetric arginine dimethylated proteins in the immunoprecipitated fraction of the WT compared to *PRMT2*$^{-/-}$ BMDMs. We interpret these as direct PRMT2 substrates. Surprisingly, we also found 80 arginine dimethylated proteins present in the *PRMT2*$^{-/-}$ BMDMs that were not present in WT BMDMs. We interpret this as PRMT2 negatively regulating another asymmetric arginine dimethylation enzyme. We also identified a set of proteins (268) that were present in both WT and *PRMT2*$^{-/-}$ BMDMs and interpret these as substrates modified by an arginine dimethylation enzyme other than PRMT2. We are focusing our efforts on charactering the direct PRMT2 substrates.

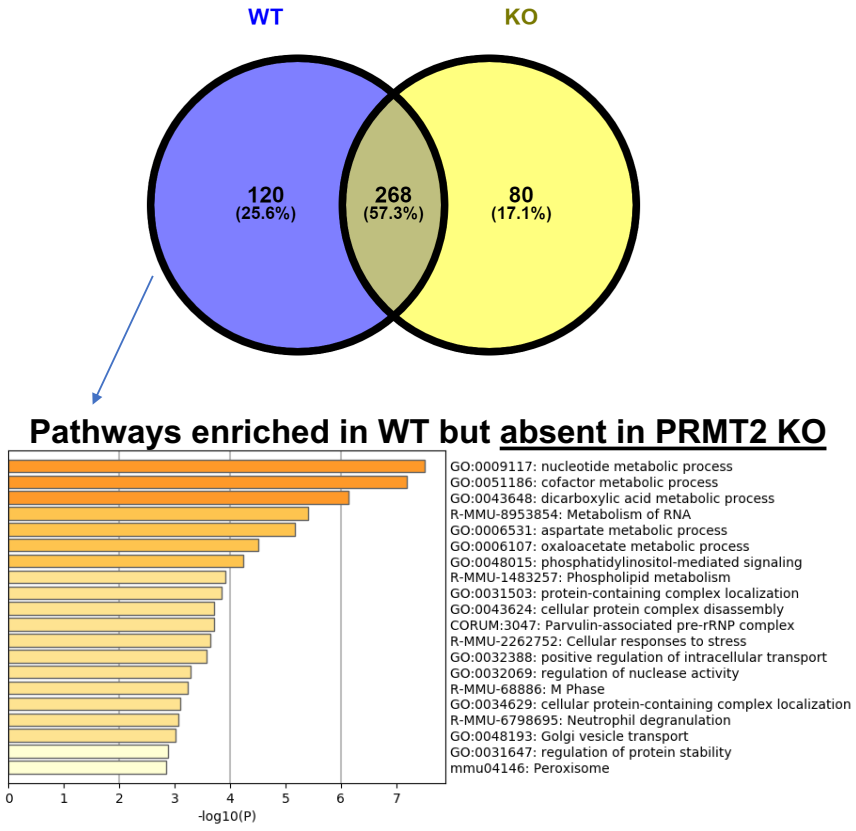


Figure 5. PRMT2 substrates and functional classes. WT and *PRMT2*$^{-/-}$ BMDMs were lysed and proteins were affinity purified with an asymmetric arginine dimethylation antibody and proteins identified by mass spectroscopy. GO classes for the substrates enriched in WT but absent in *PRMT2*$^{-/-}$ are shown.

Pathway analysis using Metascape showed that proteins enriched in WT but absent in *PRMT2*$^{-/-}$ BMDMs were associated with GO classes that include nucleotide metabolic processes. We are in the processes of validating the top asymmetric arginine demethylated substrates identified by mass spectrometry, and once validated we will begin to test their functional significance.

Major Task 2: Mapping PRMT2 cis regulatory elements by reporter gene assays based on ENCODE data

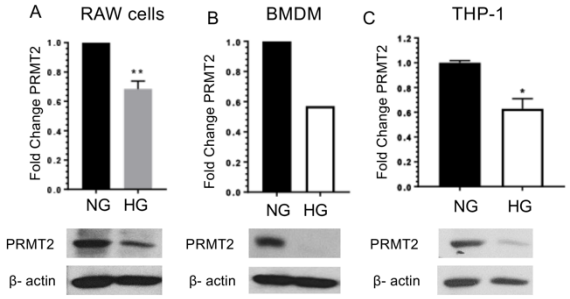


Figure 6. Reduction of steady state PRMT2 mRNA and protein in high glucose. A) RAW, B) BMDMs, C), THP1 cells were cultured in normal glucose (NG) and high glucose (HG) for 1 week, and PRMT2 expression levels were measured by qPCR and normalized to cyclophilin. Protein extract were prepared and blotted for PMRT2, and beta-actin as a loading control.

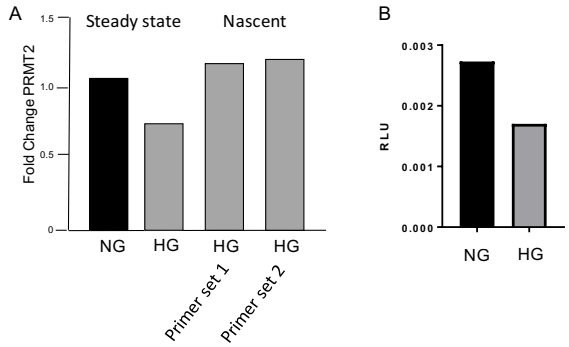


Figure 7. Post transcriptional regulation of PRMT2 mRNA. A) Steady state and nascent RNA of PRMT2 was measured by qPCR from BMDMs cultured in normal glucose (NG) and high glucose (HG). Whereas the PRMT2 steady state mRNA level is reduced in cells cultured in high glucose compared to normal glucose, the nascent RNA is not. This is true for two independent primer sets that measures nascent PRMT2 RNA. This indicates that the reduction of PRMT2 mRNA is not at the level of transcription initiation. B) PRMT2-3' UTR-luciferase reporter construct was transfected into 293 cells in normal and high glucose and activity measured and presented as relative luciferase units (RLU). Note the reduction in luciferase reporter activity in high glucose, suggesting that the decrease in PRMT2 mRNA is via the 3' UTR.

Subtask 1: Test PRMT2 promoter luciferase construct for glucose regulated expression. **(in progress)**

We have shown that the steady state PRMT2 mRNA and protein levels are reduced in high as compared to low glucose in the murine macrophage RAW cell line, both in mouse BMDMs and in human macrophage cell line THP-1 (Fig. 6). However, we have recently found that PRMT2 nascent RNA, a surrogate for newly transcribed RNA and measured by using primer pairs that span an intron-exon junction, is not reduced when cells are cultured in high glucose (Fig. 7A). This suggests that the effect of glucose is not at the level of transcription initiation, but rather regulated post-transcriptionally. Consistent with this idea, the PRMT2 promoter linked to a luciferase reporter gene did not show a change in luciferase activity in cells cultured in low and high glucose. Thus, the PRMT2 promoter alone is not sufficient to confer glucose regulation on PRMT2 expression (shown in the previous progress report).

Given that the glucose-dependent regulation of PRMT2 appears to be post-transcriptional, we examined whether the PRTM2 mRNA 3'UTR was a potential target regulation. To do this, we used a luciferase reporter construct fused to the 3' UTR of PRMT2 compared to a reporter without the PRMT2 3' UTR. We found a reduction in expression of the luciferase reporter in high compared to normal glucose for the PRMT2 3' UTR construct compared to control (Fig. 7B), suggesting that the high glucose-dependent decrease in PRMT2 mRNA is controlled via its 3' UTR.

- **What opportunities for training and professional development did the project provide?**

Nothing to Report.

- **How were the results disseminated to communities of interest?**

We will disseminate our findings through publications in peer reviewed journals, although at this point we have nothing to report.

• **What do you plan to do during the next reporting period to accomplish the goals and objectives?**

Our goals for the next reporting period are to complete the analysis of the PRMT2-/- mice under regression conditions as a function of diabetes, and to examine macrophage trafficking and changes in gene expression by RNA-seq from laser captured micro dissected CD68+ macrophages. Depending on the outcome and from the bulk RNA seq study from laser captured micro dissected CD68+ macrophages from the plaque, we will then perform validation studies of factors and pathways so identified.

We will validate the top the asymmetrically dimethylated substrates identified by mass spectrometry, and once validated we will begin to test their functional significance.

We will also continue to elucidate the mechanism whereby expression of PRMT2 is controlled by high glucose, focusing on the post-transcriptional regulatory mechanisms.

4. IMPACT:

Our work will determine the impact of PRMT2 in atherosclerosis upon diabetes. We also predict that our proteomic analyses of PRMT2 substrates will be useful in elucidating the mechanism of PRMT2 function in macrophages, and in identifying possible interventions and biomarkers. Finally, understanding the regulation of PRMT2 will enable us to develop ways to restore the normal level of PRMT2 in diabetes, and prevent the cells from contributing to plaque formation.

What was the impact on technology transfer?

None at this point

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What was the impact on society beyond science and technology?

None at this point

5. CHANGES/PROBLEMS:

We will modify aim 3, as the glucose dependent regulation appears to be not at the level of transcription initiation but rather post-transcriptionally via the PRMT2 3 UTR. Also, because of the almost one year delay in working out the mouse protocol details, we are still behind our original time table, but as summarized above, we have made significant gains and we expect to complete the study.

6. PRODUCTS:

Nothing to Report

7: PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

PIs

Name: Michael Garabedian, PhD.
Project Role: PI.
Nearest person month worked: 3
Contribution to Project:
Dr. Garabedian helped design and analyze the experiments involving the identification of the PRMT2 substrates and determining the regulation of PRMT2 expression by high glucose.
Funding Support: CDMRP/NIH

Name: Edward Fisher, MD, PhD
Project Role: Partner PI.
Nearest person month worked: 2
Contribution to Project:
Dr. Fisher helped design experiments involving the role of PRMT2 in the regression of atherosclerosis and diabetes.
Funding Support: CDMRP/NIH

Post-doctoral fellows
Prashanth Thevkar Nagesh, PhD
Project Role: Post doc
Nearest person month worked: 9
Contribution to Project:
Dr. Nagesh is performing the experiments involving the PRMT2 substrates identification and the PRMT2 gene regulation studies.
Funding Support: CDMRP/NIH

Beyza Vurusaner Aktas, PhD
Project Role: Post doc
Nearest person month worked: 9
Contribution to Project:
Dr. Aktas is performing the experiments involving the impact of the loss of PRMT2 expression in the regression of atherosclerosis in diabetes.
Funding Support: CDMRP/NIH

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

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8 SPECIAL REPORTING REQUIREMENTS: COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.

An independent report will be submitted by Dr. Fisher, the partnering PI.

9 APPENDICES: none

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