Modulating Physiologic Effects Of Phospholipid Metabolism In Obesity And Diabetes

AIM 4: Composition and Function of Sarcoplasmic Reticulum in persons with the Metabolic Syndrome

Comp-SR
Version 6
HRPO201311099
01/FEB/2021

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ROLE OF SARCOPLASMIC RETICULUM IN INSULIN SENSITIVITY AND THE METABOLIC SYNDROME

INTRODUCTION, BACKGROUND AND SIGNIFICANCE

Global death rates fell for most diseases over the last two decades. Diabetes is a glaring exception, with a 93% increase in the absolute number of deaths (second only to HIV/AIDS) and a 70% increase in global years of life lost between 1990 and 2010 [1]. With at least 1.5 billion overweight people in the world, a third of whom are obese [2], diabetes and premature death are certain to accelerate. While exercise has been shown to prevent diabetes and is a proven treatment for the metabolic derangements that occur in diabetes[3], persons with the metabolic syndrome, pre-diabetes and diabetes often have poor exercise tolerance[4]. While overall lack of fitness is multi-factorial, reduced exercise performance may be related to fuel metabolism in skeletal muscle in susceptible individuals, including those with diabetes[5-7]. Exercise has been shown to improve metabolism of glucose and reduce insulin resistance in skeletal muscle, but the role of lipid metabolism to insulin sensitivity and exercise performance is not well understood. This study is a pilot that will explore the role of lipid metabolism in key metabolic pathways in skeletal muscle. The overall goal is to understand the abnormalities that occur in lipid metabolism in sedentary persons with the metabolic syndrome compared to controls without the metabolic syndrome who are normal weight and sedentary. This study will add to our understanding of how lipid metabolism contributes to insulin sensitivity and function in skeletal muscle.

The sarcoplasmic reticulum (SR) is the muscle cell's production and function center. It is made mostly of a phospholipid (fatty) layer that is also integral to its function. Fatty acid synthase (FAS) and choline/ethanolamine phosphotransferase 1 (CEPT1) are two proteins involved in the production of this phospholipid layer. Depending on the activity of these proteins, this phospholipid layer can assimilate more of certain types of phospholipids, mainly phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Phosphatidylethanolamine is the phospholipid species assimilated when FAS and CEPT1 are active. The more active these proteins, the more PE is present in the SR layer. The main alternative to PE is phosphatidylcholine. More of this phospholipid species is assimilated into the SR when the above proteins are not active. Each of these phospholipids carries a different structure and with it, different functional properties, so depending on which type of phospholipid is more abundant, the overall structure and function of the SR is varied.

Interestingly, our new data show that high fat feeding leads to increased fatty acid synthase activity in mice. In humans, we have found that FAS is increased in the skeletal muscle of obese, yet healthy subjects, while it is decreased in metabolically compromised obese subjects (Fig 1). Furthermore, we have found that surgically induced weight loss decreases both FAS and CEPT1 (Fig 2). These results suggest that FAS and CEPT1 are part of a phospholipid production pathway brought on by high fat feeding or obesity to preserve the function of the sarcoplasmic reticulum. This fails in those who progress to metabolic decompensation (the obese 'abnormal' in Fig 1A).

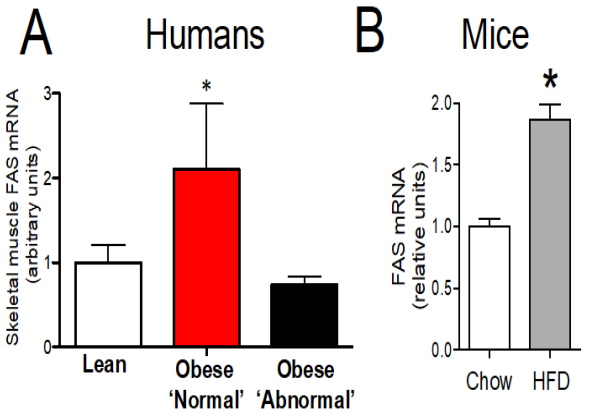

Fig 1. FAS mRNA levels in human skeletal muscle. *P<0.05 vs. obese abnormal by Tukey's post hoc test. B. FAS mRNA in mice from Fig 1A of reference 4.

Given the function of these proteins, these studies suggest that the phospholipid composition may also be altered in metabolically compromised individuals when compared with normal subjects. Indeed, this hypothesis was supported by our findings of altered phospholipid composition in mice with artificially lowered activity of these proteins. There was more PC than PE in the sarcoplasmic reticulum of these mice when fed a high fat diet.

But what does the sarcoplasmic reticulum have to do with blood sugar control? It turns out that insulin sensitivity is related to the activity of a protein that interacts with the sarcoplasmic reticulum, called sarco/endoplasmic reticulum ATPase (SERCA). The main function of SERCA is to store calcium in the sarcoplasmic reticulum in order to allow the muscle to relax. This resets the muscle to allow for repeated muscle contraction. However, SERCA is only functional when actively associated with the phospholipid layer of the SR. Calcium also seems to regulate insulin sensitivity in the muscle cell through the proteins CaMKKβ, AMPK, and AS160 (Fig 4). When SERCA is not active and therefore calcium is not stored in the SR, muscle cells are more insulin sensitive. When SERCA is active and therefore calcium is stored away and not available in the cytosol of the cell, muscle cells are more insulin resistant.

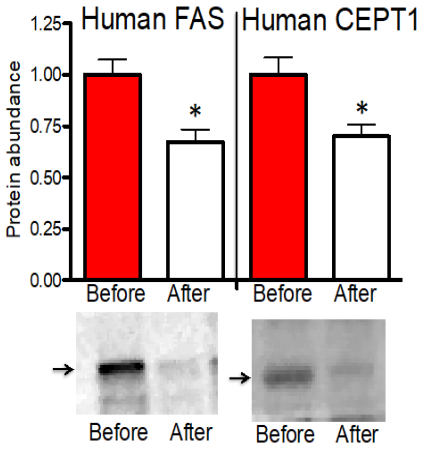

Fig 2. FAS and CEPT1 protein in skeletal muscle biopsies from 16 obese human subjects before and after 20% weight loss induced by gastric bypass.

How do these findings relate to the composition of the sarcoplasmic reticulum? Our studies have shown that SERCA has lower activity when the composition of the sarcoplasmic reticulum is comprised of more PC than PE. Mice that are artificially induced to have this altered SR composition through inactivation of FAS are found to have muscle weakness, but this is in exchange for improved blood sugar control. In normal mice with functioning FAS, the PE to PC ratio is preserved and muscle strength is normal, but at the cost of reduced insulin sensitivity. This suggests that insulin sensitivity may be determined by the ratio of PC to PE in muscle tissue, based upon the activity of SERCA. When there is more PC than PE, the activity of SERCA is lower and thus muscle function is compromised, but the cell's sensitivity to insulin is higher. Conversely, when there is more PE than PC, the activity of SERCA is higher and thus muscle function is preserved, but the cell's sensitivity to insulin is lower. Our conclusion from these observations is that the muscle cell sacrifices insulin sensitivity for muscle function when called upon to do so. The muscle cell's first priority is to contract, not to be sensitive to insulin.

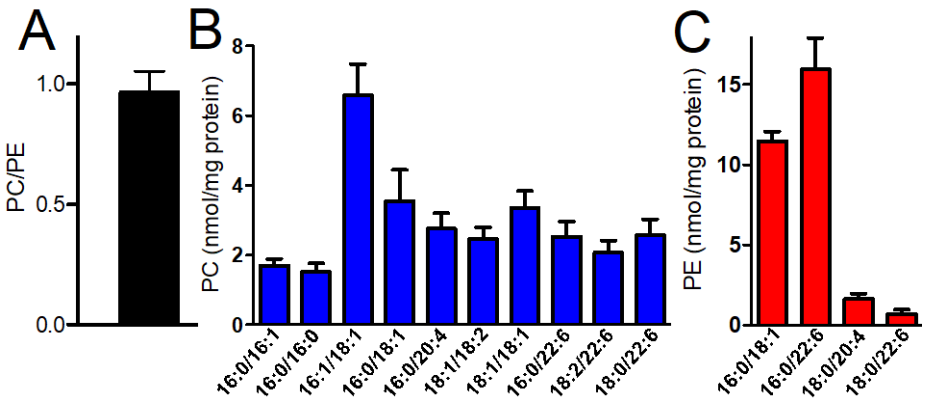

Fig 3. A. Human muscle PC/PE ratio (0.97±0.088). B. PC species. C. PE species. As in mouse, 16:0/22:6 is the most abundant PE species in human muscle.

But does this insulin-desensitizing phospholipid ratio apply to humans? For example, we know that FAS and CEPT1 activity apply to humans, but does the activity of these proteins affect the ratio of PC to PE in humans as well? To see if the findings in mouse muscle can translate to human muscle, we analyzed skeletal muscle biopsies from four humans. We found that the human sarcoplasmic reticulum phospholipid composition was similar to that of the sarcoplasmic reticulum from mice (Fig 3). These results established the feasibility for studying the muscle of humans with the metabolic syndrome.

If indeed we do find that insulin sensitivity in humans can be predicted by the phospholipid ratio in skeletal muscle, this would verify the involvement of phospholipids in insulin sensitivity. This study will help to create a new context in our understanding how diabetes works, and how we as a medical community can combat it. Specifically, therapy could be guided by utilizing non-invasive imaging, such as MRI to identify specific skeletal muscle phospholipid signatures. Drugs could be developed or even repurposed to alter the structure and function of the sarcoplasmic reticulum and offer new options for the treatment of obesity and diabetes. This would be paradigm shifting in our understanding of how muscle contributes to overall insulin resistance or sensitivity, and would pave the way to further studies and possible therapeutics that target muscle in ways not previously thought of.

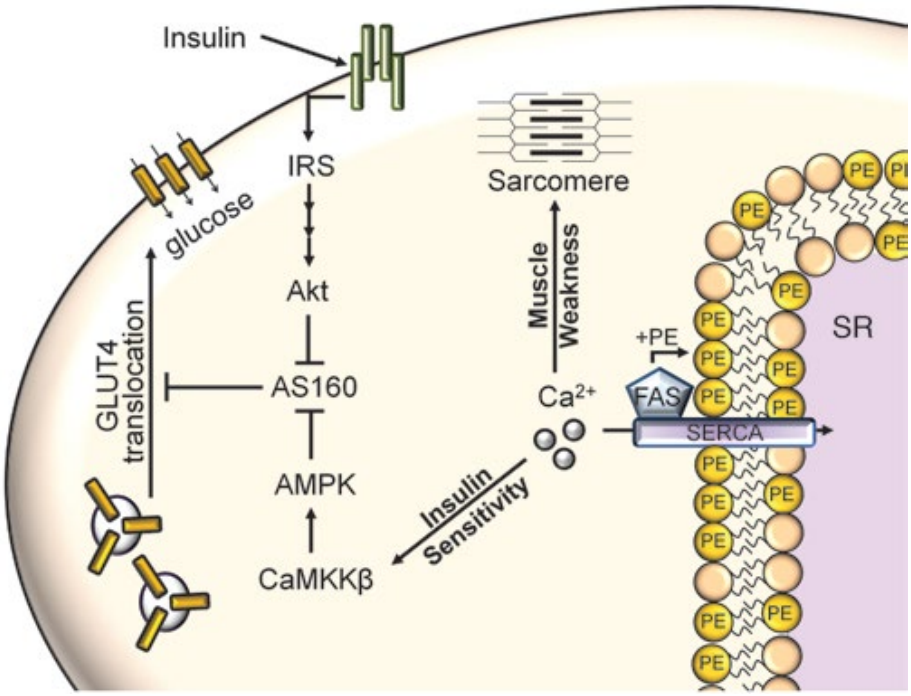

Fig 4. Increased cytosolic calcium after FAS inactivation likely impairs relaxation of the actin-myosin junction at the sarcomere, promoting muscle weakness. Calcium also likely activates a signaling cascade involving CaMKKβ, AMPK, and AS160 that enhances insulin-stimulated glucose uptake in muscle. (Taken from reference 5)

SPECIFIC AIMS

The specific aims of this study are to determine if the composition and function of the sarcoplasmic reticulum is altered in persons with the metabolic syndrome compared to lean controls. Long-term goals are to deliver new understanding of impediments to effective therapy, novel biomarkers of disease progression, and innovative treatment targets for diabetes.

PROTOCOL PLAN

Study Design. We will perform a cross-sectional study of age-matched, sedentary lean controls and individuals with the metabolic syndrome, recruited in part from a large database of subjects screened for our recently completed clinical trial (manuscript in preparation) involving metabolic syndrome (NCT00455403).

Eligibility Criteria

Control subjects

Inclusion:

1. Sedentary: No routine exercise; walking <10,000 steps per day
2. Age >18 and <65
3. Body Mass Index (BMI) <32
4. Healthy by physical exam (patient is devoid of major acute or chronic illness)
5. No significant abnormality in screening labs

Exclusion:

1. Currently on any significant prescription medications other than oral contraceptives in women
2. Currently Pregnant
3. Current Tobacco use

Subjects with the Metabolic Syndrome

Inclusion

1. Sedentary: No routine exercise; walking <10,000 steps per day
2. Age >18 and <65
3. Body Mass Index (BMI) >30
4. Meet at least three of the following ATP III criteria for the metabolic syndrome:
    a. waist circumference > or = 40 inches in men, > or = 35 inches in women
    b. blood triglycerides > or = 150 mg/dL
    c. blood HDL cholesterol <40 mg/dL in men, <50 in women
    d. blood pressure > or = 130 mmHg systolic, or > or = 85 mmHg diastolic
    e. fasting blood sugar > or = 100 mg/dL

Exclusion

1. Diagnosed with Type 2 diabetes, coronary artery disease, cancer, liver, lung, or kidney disease or any other major illness
2. Currently on any significant prescription medications other than:
    a. oral contraceptives in women
    b. More than two standard medications for stage 1 hypertension in men or women (blood pressure 140-159/90-99)
3. Currently Pregnant
4. Current Tobacco use

Study Plan and Procedures

*Screening***.** Potential subjects will be interviewed, the protocol will be explained in detail, then risks and benefits of involvement will be discussed. Those subjects interested in participating will be given ample time to review the informed consent, to ask questions of any member of the study team, and will sign the informed consent prior to any study procedures being conducted. Subjects will have a complete history and physical examination (including measurement of blood pressure according to a standard protocol and determination of height, weight and waist circumference, all performed by experienced personnel in the Washington University IRU) or the study coordinator. A CBC, CMP, HBA1c, and a lipid panel will be performed to determine whether the patient meets the inclusion criteria and does not meet any exclusion criteria. Individuals who have one or more exclusions will be informed of their test results but will not proceed further. If the patient meets the inclusion and does not meet the exclusion criteria specified above at the screening visit, he or she will be scheduled for three subsequent visits, as depicted in the schedule of events.

Rescreening Visit. If potential subject fails screening based upon a single criterion, i.e. a lab result or blood pressure that is thought not to be reflective of prior results based on history, at the discretion of the investigator the screening procedure may be repeated once.

*Study procedures***.** Patients who provide informed consent, and who meet the inclusion criteria and do not meet the exclusion criteria will received 2 phone calls (Visits 2 and Visit 3) approximately 1 week apart and will return to the study site for a final subsequent visit. At visits 2 and 3 study staff will be verifying no change in eating or exercising habits, as well as no change in medications or health status. Visit 4 will interview subjects to determine no changes in

eating or exercising habits, no changes in medications or health status and the following procedures: lab work (HBA1c, Lipid panel, fasting glucose and insulin levels, as well as 3 vials of blood for future use), an oral glucose tolerance test including an insulin level with each draw, a DEXA scan, and a muscle biopsy. The schedule of events, as well as explanations of each procedure is outlined below.

Comp-SR Schedule of Events					
Procedure	**Screening**	***Rescreen**	**Phone visit**	**Phone visit**	**Final visit**
	Week 1		Week 2	Week 3	Week 4
Fasting (10-12 hours)	X	X			X
Informed consent	X				
H&P	X				
Vital signs (BP, HR, Temp)	X	X			X
Weight	X	X			X
Waist circumference and Height	X				
Urine Pregnancy test in women of childbearing potential	X	X			X
Verify no change in exercise		X	X	X	X
Verify no change in eating habits		X	X	X	X
Verify no change in medications or health status		X	X	X	X
CBC, CMP, HBA1c, Lipid panel	X	X			
HBA1c, Lipid panel, fasting glucose and insulin levels, as well as 3 vials of blood for future use (All drawn 5 min prior to OGTT)					X
OGTT: 0, 30, 60, 90, 120 minutes with insulin levels also drawn at each time marker					X
DEXA scan					X
Leg Muscle biopsy					X

***Subject will only have the failed test repeated.**

Blood tests. Most determinations will be performed by the Washington University Core Lab for Clinical Studies, a CLIA-certified facility that has served as the reference lab for landmark clinical trials including the Coronary Primary Prevention Trial and the CARE trial. The lab will measure CBC, CMP, HBA1c, and Lipid panel at first visit and HBA1c, Lipid panel, and glucose and insulin levels, as well as 3 more vials of blood for future research at the final visit.

Oral Glucose Tolerance Test (OGTT). This test will be performed on the final visit. Study patients will be asked to arrive fasting for 10-12 hours ahead of time. An intravenous catheter will be placed, labs including Hemoglobin A1C, lipid panel, and blood glucose and insulin levels will be obtained 5 minutes prior to the start of the OGTT. Blood will be drawn for blood glucose and insulin levels at the start of the OGTT. The patient will then be asked to consume 75 grams of glucose. Blood will be drawn again for blood glucose and insulin levels at 30, 60, 90 and 120 minutes after consuming the glucose. This visit will take approximately 4-6 hours.

DEXA scan. This test will be performed on the final visit. We will ask the patient to lie on a table while they are scanned by a special machine. The DEXA scan takes about 30 minutes. DEXA images taken during the study are for specific research purposes and are not being used to

evaluate the patient's health or find medical abnormalities. These images will not be reviewed by a radiology physician to diagnose existing abnormalities.

Muscle Biopsy. This procedure will be performed on the final visit. A member of the study team will obtain a sample of muscle tissue from the patient's thigh (vastus lateralis muscle). This involves numbing the skin with a local anesthetic (lidocaine), making a small (approximately the size of the dotted line ------ ; 1 cm) incision and removing a small (approximately 1/15th of an ounce) piece of muscle tissue. Each incision will be closed with a piece of sterile tape or a stitch, if necessary.

Sample Size Estimates and Statistical Analyses

There are no published data to guide estimates; to our knowledge, this type of study has never been done. If SERCA activity is 25% less in the metabolic syndrome, based on a standard deviation of 0.445 (Fig 14), 17 subjects per group are required (80% power, α=0.05). We will enroll 25 for each group to account for assay problems and to increase power. For the mouse and cell culture studies, primary endpoints are clearly defined. Differences and variances from the first experiments will be used to estimate sample size (80% power, α=0.05). All comparisons will use appropriate tests for parametric and nonparametric data. Suitable posttests will be used for multiple groups.

Anticipated Results, Alternative Approaches, Potential Problems and Benefits

Anticipated results of the study are lower SERCA activity and higher SR PC/PE ratio in people with metabolic syndrome compared to lean controls. We expect to find higher FAS and CEPT1 in metabolic syndrome skeletal muscle, reflecting activation of a phospholipid synthetic pathway attempting to replenish SR membrane composition disrupted by the obesity/high fat state.

Predicting relationships between insulin sensitivity and SERCA activity is difficult based on our mechanistic studies. The most insulin resistant could have the highest levels of FAS and CEPT1 despite lower SERCA activity in the setting of a higher PC/PE ratio. This would reflect the inability to synthesize key PE molecules due to the stress of lipid overload despite induction of the FAS-CEPT1 pathway.

An alternative approach could include euglycemic insulin clamps, but the Matsuda index is highly correlated with glucose disposal and much less invasive than a clamp.

Potential problems in this study include patient lost to follow up, rarely loss of confidentiality, drawing of blood may cause minor discomfort, bruising, bleeding, chance of infection or blood clotting associated with the insertion of the needle and rarely infection or fainting.

The patient could experience blood sugar fluctuations during the OGTT causing discomfort; sweating, shaking, nausea. However, blood sugar is carefully monitored throughout the OGTT to decrease the risk.

Risk associated with the placement of catheter is similar to blood drawing, infection or injury from the muscle biopsy, reaction to the local anesthetic (lidocaine/xylocaine), or exposure of a developing fetus to the DEXA scan. Careful precautions will be taken and proper procedures are in place to avoid these complications, participants are under constant medical supervision (i.e. proper training of staff and physicians to perform blood drawing, muscle biopsies, questioning subject about prior allergic reaction to lidocaine/xylocaine, pregnancy tests prior to DEXA scanning and review of medical history, including history of radiation exposure for research testing.)

To protection patient confidentiality all data, forms, and specimens will be labeled with each study participant's unique study identifier. The optional stored biologic samples are coded (with initials, DOB, gender & race) and stored in a locked lab with access to study staff. Biologic

samples will not have identifiers but only a study code - the lab will not have access to the link. Only Washington University study staff will have the link between the biologic sample code and the subject identifiers.

Stored Samples will be kept at the Washington University Core Lab for Clinical Studies and/or Dr. Clay Semenkovich's lab. Access to the storage area is restricted by pass card swipe; samples are kept in a locked room and in a locked cabinet. Only authorized staff will have access to the samples. Electronic stored data (may contain subject initial and/or DOB) and study identifier maintained on WUSM secure computer network on a password protected database with access restricted to designated study staff only. Any information maintained in electronic format that includes identifiable information will be stored on the secure network, password protected and restricted to WU study staff only.

Study source documents will contain subject name, date of birth and other identifying information. Study data collection documents may contain subject identifier (initials and/or assigned #). Subject identifiers will be collected and maintained for internal (WU) use only. Confidential binders and study files with the subject info will be kept in a restricted access area in a locked office and a locked cabinet when not in the possession of study staff.

Withdrawal of research subjects from the protocol may occur. Research subjects will be withdrawn if any of the following occurs:

- The patient withdraws consent for any reason
- An adverse event occurs related to study procedures (i.e. severe infection from muscle biopsy)
- An adverse event that would impact any of the study results occurs. An example would be a patient who develops poison ivy during the study and requires oral steroids for control of the rash. This would disrupt their weight steady state as steroids are known to cause weight gain. Efforts will be made to adjust the study schedule to permit recovery, but if that is not possible or likely, the patient will be withdrawn.

The PI and/or sub-I will monitor each subject's lab results and visit data at each study visit.

Possible benefits to study patients would be satisfaction of altruistic values alone.

Adverse Event

An Adverse Event (AE) is any untoward medical occurrence in a study participant regardless of its relationship to study treatment.

A Serious Adverse Event (SAE) is any untoward medical occurrence that results in death, is life-threatening, requires hospitalization or prolongation of an existing hospitalization, results in persistent or significant disability, or is a congenital anomaly/birth defect. Important medical events that do not fall into the above categories may also be considered an SAE when, based on medical judgment, such events may jeopardize the patient's safety and require medical/surgical intervention to prevent one of the outcomes listed in the SAE definition. The term SAE is not intended as a measure of severity or intensity.

Adverse Events Reporting

All AEs will be reported on the Adverse Events form that will be completed by the study staff at each regular follow-up visits. Forms will include standardized questions relating to specific events of import in patients as well as any significantly abnormal physical finding identified on examination and any significantly abnormal laboratory results obtained on the patient between visits or at the time of the visit. AEs reported or ascertained between clinic visits will be captured and reported at the time of the next schedule visit. Pre-existing conditions (that is, conditions present prior to randomization) will not be considered or recorded as AEs unless the

condition worsens in intensity or frequency after randomization. Likewise, continuing AEs will not be reported as AEs at subsequent visits unless they increase in severity or frequency between visits, they results in criteria for a SAEs, and/or they resolve between visits. The study staff will report all AE's to HRPO according to its AE reporting policy and procedures.

Assessment of Causality and Severity

The seriousness of adverse events will be ascertained by investigator and the need for further evaluation, follow-up, or referral. The relationship between study participation and AEs will be determined according to the following criteria:

Not related – temporal relationship of the onset of the event, relative to study participation, is not reasonable or another cause can by itself explain the occurrence of the event.

Possibly related – temporal relationship of the onset of the event, relative to study participation, is reasonable but the event could have been due to another, equally likely cause.

Probably related – temporal relationship of the onset of the event, relative to study participation, is reasonable and the event is more likely explained by the study treatment than by another cause.

Definitely related – temporal relationship of the onset of the event, relative to study participation, is reasonable and there is no other cause to explain the event.

Study Data Monitoring and Safety

The study will be reviewed for safety, privacy and data integrity by the investigator(s) at regular intervals during the course of the study and at least annually by a designated Safety Officer

The purpose of the safety officer's oversight is to evaluate accumulating data and provide continual safety oversight of trial subjects and recommendations. The Safety Officer will review data annually with ad hoc meetings reviews as necessary. The purpose of the annual review is to review safety information, to review factors relating to quality of trial conduct, and to ensure proper implementation of procedures for the trial.

The Safety Officer will be an independent, senior faculty member having pertinent expertise in the management of patients in the conduct and monitoring clinical trials and free from interest conflicts. The Safety Officer will submit a written report of patients reviewed, any pertinent findings, and decision about continuation or stopping the study annually. The report will be submitted to the Human Research Protection Office.

Data Collection and Quality Assurances

In accordance with applicable regulations, GCP, and HRPO requirements the PI will review with the study staff the protocol, study requirements, and their duties and responsibilities to satisfy regulatory, ethical, and HRPO requirements.

Study personnel will enter demographic data and study results into a database and/or spreadsheet for analysis. Subjects will be de-identified and assigned a subject number for the analysis data set. Information entered into databases will be stored on a secured server, which is password protected and accessible only to authorized study staff.

When reviewing data collection procedures, the discussion will include identification, agreement, and documentation of data items for which a paper chart will serve as the source document. The PI and sub-Is, will monitor the study to ensure that the:

- Data are authentic, accurate, and complete
- Safety and rights of subjects are being protected
- Study is conducted in accordance with the currently approved protocol, GCP, and all applicable regulatory requirements. (Faculty sponsor is only when the PI is in training.)

Subjects will be requested to contact study staff if any adverse event occurs between clinic visits. All adverse events, whether incurred at a study visit or between study visits, will be reviewed by PI, and submitted to HRPO in accordance with HRPO policy. The PI or Dr. McGill will remove any subject at his or her discretion based upon his judgment or in the event of undue AE's. New risks will be added or the risk language adjusted in the informed consent per HRPO policy and participants will be notified of any and all changes, in particular those that might affect their decision to participate.

Safety and tolerability assessments include the following:

- AEs and SAEs
- Physical examinations, laboratory evaluations, vital sign measurements (blood pressure and pulse rate)

All safety data will be available in data listing in the clinical protocol report. Data will be described in terms of descriptive statistics reviewed on a continuous basis by study staff and the Safety Officer. The number of patients screened, enrolled, and completing the study will be summarized.

Study safety will be monitored by PI and sub-Is. PI will review all reported AE's and report to HRPO, as per HRPO requirements. The PI may halt the study participation or the study if determined to be in the best interest of participants. Safety Reports will be generated ad hoc and reported as per HRPO policy and at least annually at the time of HRPO renewal. Since this study does not involve an intervention, i.e., is purely observational, a DSMB will not be convened.

Study Termination

The study will be terminated if it is determined that continuing the study would pose an unacceptable risk to subjects.

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