Cognizance Biomarkers, LLC

3160 Chestnut St
Suite 200
Philadelphia, PA 19104-2853

Biomarker Protocol:

Proof of Principle for a Diagnostic Blood Test of Recurrent Seizures

Study Center:

University of Pennsylvania

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Contents

1 Background & Rationale

The CDC estimates that approximately 10% of the US population will experience a seizure and 3% will be diagnosed with recurrent seizures (epilepsy) before age 80. Unfortunately, diagnosing epilepsy is often a long, costly, and challenging process involving a combination of a physician's clinical impression, electroencephalogram (EEG), magnetic resonance imaging (MRI) and, where available, a multiday inpatient test that combines closed circuit TV and EEG (CCTV/ EEG monitoring). Due to both the high cost and complexity of diagnosis, approximately 30% of patients who are treated by a neurologist for epilepsy are actually experiencing events from other causes, such as syncope, transient ischemic attack, or psychiatric disorder.[1] A rapid, cost-effective, and accurate blood test for epilepsy would allow clinicians to prescribe treatment quickly for patients who test positive and to redirect the workup on patients who test negative. While there have been many previous efforts to find such a blood test, including tests of hormone concentrations such as prolactin and tests of brain tissue breakdown such as neuron specific enolase, [2, 3] these have not had a high enough sensitivity or specificity for epilepsy to be of clinical value.

Recent studies have established that brain inflammation is a prevalent etiological process factor in focal epilepsies, and may contribute to seizure generation.[4] Levels of pro-inflammatory cytokines are increased in epilepsy surgical pathology specimens, which likely elevate levels of excitatory neurotransmitters and impair neuronal inhibition, increasing the likelihood of seizures.[5] Further, blocking this inflammation reduces or prevents seizures in preclinical models.[6]

Consistent with this research, investigators at the University of Pennsylvania have identified a potential diagnostic blood test for epilepsy called the CNS-specific Assay for Recurrent Paroxysmal Events (CARPE), which is defined as the ratio of the plasma concentrations of two lymphocytic regulatory proteins: TARC and sICAM5. The CNS-specific component of this test is the soluble fraction of Intracellular Adhesion Molecule 5 (sICAM5), a protein that likely originates in the CNS. Preliminary data show that anti-inflammatory sICAM5 concentrations are consistently low in epilepsy patients. The second component of the test is Thymus and Activation Regulated Chemokine (TARC or CCL17), a lymphocyte chemoattractor that in preliminary data was present in consistently high concentrations in epilepsy patients. In contrast with the T-cell inhibitory function of sICAM5, TARC binds specifically to CCR4 receptors on T-cells and induced chemotaxis in T-cell lines. TARC is known to be principally expressed peripherally in blood mononuclear cells and the thymus.[7, 8] The origin of the high concentrations of TARC found in patients with seizures is not known and could arise either systemically or within the central nervous system. Nonetheless, TARC concentrations are high in patients with seizures, consistent with a pro-inflammatory process.

The present study is designed to establish the accuracy of CARPE as a diagnostic tool for epilepsy in an outpatient neurology clinic, the population that would most immediately benefit from such a test. Exploratory goals include determining whether additional cytokines and chemokines may enhance CARPE's accuracy, determining whether levels of sICAM5 or other cytokines or chemokines predict or confirm subjects' responses to antiepileptic drugs (AEDs), and determining the degree to which CARPE's accuracy is temporally related to seizures or seizure-like events.

2 Study Design

Specific Aim 1: To establish the accuracy of the CARPE blood test for determining the presence of epilepsy, and in doing so, establish the threshold for diagnosis

a. Hypothesis: CARPE will accurately identify greater than 70% of patients as epileptic

b. Method 1a: Enroll a total of 240 Research Subjects aged 12 and older who have been referred to the University of Pennsylvania's Penn Epilepsy Center for diagnostic evaluation and have a subsequent workup at Penn or an outside site that includes an EEG. All subjects will be asked to provide a single sample of blood for CARPE testing and allow the study team to review their medical records and collect information about their epilepsy diagnosis. Subjects who enroll and do not give a blood sample and have an EEG that is available for study review will be replaced

   Analyzed subjects' clinically indicated work-up must include an EEG, and additional diagnostic tests will be at the discretion of the subjects' physicians. After six months of clinical care, an independent panel of three epileptologists will review the subjects' medicals records and determine each subject's diagnosis. The study team will compare the panel's diagnoses with the CARPE results

c. Method 1b: Collect up to two 5ml tubes of blood via a single venipuncture from a maximum of 240 individuals aged 18 and over who accompany patients to their Penn Epilepsy Center appointments as true controls. To be eligible, Control Subjects must confirm that they have not been diagnosed with epilepsy and that they are not taking any medications used to treat epilepsy, and they will be asked to sign a separate Informed Consent Form. As explained in the ICF, the study team will not collect any PHI from Control Subjects; information collected will be limited to the following data points at the time of consent: age, gender, presence of an epilepsy diagnosis, and whether they are taking any medications used to treat epilepsy (potential subjects will be provided with a comprehensive list with brand and generic medication names to review).

Specific Aim 2: As an exploratory endpoint, we will determine whether additional cytokines and chemokines may enhance the accuracy of the CARPE

Specific Aim 3: As an exploratory endpoint, we will determine whether CARPE's diagnostic accuracy is temporally related to time of last seizure

a. Hypothesis: CARPE's accuracy decreases as more time elapses between the seizure or seizure-like event and sample collection

b. Method: Collect postictal blood samples from Research Subjects aged 12 and older who have been referred to the University of Pennsylvania for continuous video EEG (cVEEG) monitoring. Subjects will be asked to provide a single sample of blood for CARPE testing per day and up to one additional blood sample per day (12:00am-11:59pm) if the subject experiences a seizure or seizure-like event while undergoing cVEEG.

   We will use clinical determination as the gold standard for classifying the event.

Specific Aim 4: As an exploratory endpoint, we will determine whether levels of sICAM5 or other cytokines or chemokines predict or confirm AED efficacy

a. Hypothesis: CARPE levels will distinguish Research Subjects who achieve seizure freedom, defined seizure freedom since treatment change

b. Method: Research Subjects who join the study will have the option of providing a sample of blood for research at any regularly scheduled clinic visit that takes place during the 12 months after enrollment

3 Subject Selection, Enrollment, & Withdraw

3.1 Vulnerable Populations

Fetuses, neonates, and prisoners are not eligible to participate. Pregnant women will not be targeted but will not be excluded, provided that they have not given, and are not expected to give, blood for any other reason within one week of the study. Children under 12 are excluded; children aged 12-17 must provide assent if they are mentally capable of doing so, and must also have a caregiver provide Informed Consent on their behalf.

Potential Control Subjects must be able to provide consent on their own behalf. Research Subjects who are unable to consent for themselves, including those who are institutionalized, are eligible provided that they do not express any objections and a Legally Authorized Representative (LAR) signs Informed Consent on their behalf. Inclusion of individuals who are unable to provide informed consent is based on the principle of beneficence: approximately 25% of epilepsy patients fall into this category, and they are well-represented in the group that may ultimately benefit from this test. In all cases, every effort will be made to inform both the potential subject and the healthcare representative of the study and the associated risks and benefits.

Any member of the following classes, who is reasonably available, may act as a subject's healthcare representative (in descending order of priority):

- Spouse (unless an action for divorce is pending)
- Adult child (18 years or older)
- Parent
- Adult sibling
- Adult grandchild
- Other adult who has knowledge of the patient's preferences and values, including but not limited to religious and moral beliefs, who assess how the patient would make decisions

3.2 Inclusion Criteria

3.2.1 Research Subjects

- Aged 12 and older
- Evaluation in at least one of the following settings:
  - Newly referred to the University of Pennsylvania's Penn Epilepsy Center. These patients may not have completed any diagnostic evaluation at Penn, other than an initial physician consultation,

prior to enrollment and must agree to allow the study team to collect records regarding their diagnostic evaluations (including EEG). Diagnostic evaluations may take place at Penn or outside sites
- All patients who are admitted to the University of Pennsylvania for cVEEG monitoring

3.2.2 Control Subjects

- Aged 18 and older
- Provide verbal confirmation that they do not experience seizures or suspected seizures of any type (a single instance of febrile seizure prior to age 2 is allowed)
- Provide verbal confirmation that they have not taken any medications used to treat seizures for a minimum of two months prior to providing a blood sample
- Able to provide Informed Consent

3.3 Exclusion Criteria

3.3.1 Research Subjects

- Patients who, in the opinion of the investigator or the patient's neurologist, are unable to give Informed Consent due to mental impairment or any other reason AND do not have a LAR who can give Informed Consent on their behalf. Patients aged 12-17 (inclusive) must provide assent if they are mentally capable of doing so, and must also have a caregiver who provides Informed Consent on their behalf
- Patients who, in the opinion of their neurologist at the University of Pennsylvania, are not good candidates for donating blood for any reason

3.3.2 Control Subjects

- Younger than 18
- Experience seizures or suspected seizures of any type (a single instance of febrile seizure prior to age 2 is allowed)
- Treatment with an anti-seizure medication within two months prior to providing a blood sample
- Unable to provide Informed Consent

3.4 Recruitment

Before the research begins, the Principal Investigator will present this study to the University of Pennsylvania's Neurology Department's Patient-Oriented Research Committee.

The study team will also present the study to the attending epileptologists, resident physicians, fellows, and nurse practitioners at the Penn Epilepsy Center and ask for their advance permission to contact their new patients in the outpatient clinic, and their patients who are admitted to HUP for cVEEG monitoring. Physicians who object to the study for any reason may elect not to allow their patients to participate. Physicians and nurse practitioners may also elect to contact the study team to prohibit them from inviting individual patients to join for any reason, or to prohibit enrolled subjects from giving blood. All Research Subjects who join the study at their first outpatient visit may provide a blood sample in support of Specific Aim 1, regardless of when the blood draw occurs – at their first visit or at a later visit – since they will have been prospectively identified and enrolled as part of a true cohort.

In the interest of enrolling a true cohort and in consideration of the low-risk nature of this study, in the absence of an attending physician's objection, the study team may approach all potentially eligibly patients (provided that the physician has previously approved the study as described above).

The study team will review clinic lists and identify patients who are attending new outpatient visits at the Penn Epilepsy Center or are being admitted for cVEEG monitoring. A member of the study team may approach and consent any potential subject in the outpatient setting at any point during their visit. The study team may approach any potential subject who has been admitted for cVEEG monitoring at any point after his her or admission.

The study team will invite adults who accompany Research Subjects to participate as Control Subjects as time permits until a maximum of 240 Control Subjects have provided a single blood sample.

3.5 Informed Consent

No blood sample or medical information may be collected prior to or in absence of a subject or subject's LAR giving Informed Consent.

To begin the informed consent process, an appropriately trained member of the study team, such as the Principal Investigator, Sub-Investigator, Study Coordinator, or Research Assistant, will approach potential subjects in a private area and explain the overall study and the consent document using language that the individual can easily understand. The study team will make every effort to complete the informed consent process in a private room, but when space constraints render this impossible and potential subjects do not object, the study team may complete the informed consent process in a semi-private alcove space in the outpatient clinic normally. When all of the potential subject's questions have been answered to his or her satisfaction, he or she will be invited to sign the Informed Consent form. All subjects will receive a copy of the signed document. Original signed Informed Consent forms will be kept in the study files, in a locked office at the Penn Epilepsy Center. This process applies to both Research and Control Subjects.

3.6 Withdraw

If a Research or Control subject informs the research team either by phone or mail of his or her desire to withdraw from the study and the subject's sample or samples have not already been used for research, that subject's samples will be destroyed as outlined in the Informed Consent form. This decision will not affect results from research already preformed on the subject's samples, but no additional tests will be carried out and the study team will not access his or her medical record after notification of withdraw.

4 Risk Analysis

An appropriately trained individual will draw a single sample of approximately 5ml of blood by venipuncture no more than twice per day. Risks of venipuncture include discomfort, bruising, dizziness, and rarely fainting and infection.

The greatest risk associated with this study is the release of health records. In addition to following all HIPAA guidelines, the study team will take extensive steps to minimize this risk, as described below in Section 9, "Sample Processing and Storage," and Section 10, "Privacy and Confidentiality."

5 Safety and Adverse Events

All adverse events will be collected and reported in accordance with International Conference on Harmonization and Good Clinical Practice Guidelines.

5.1 Definitions

Adverse Event

An adverse event (AE) is any symptom, sign, illness or experience that develops or worsens in severity during the course of the study. A preexisting condition should only be considered an AE if it increases in frequency or intensity during the study.

Serious Adverse Event

A serious adverse event (SAE) is an AE that results in any of the following outcomes:

- Death
- Life-threatening
- Hospitalization or prolongation of hospitalization
- Congenital anomaly or birth defect
- Persistent or significant disability or incapacity
- Important medical event (in the investigator's judgment, the event jeopardizes the subject's health, or required intervention to prevent another serious outcome listed above)

Unanticipated Problems Involving Risk to Subjects or Others

Any incident, experience, or outcome that meets all of the following criteria:

- Unexpected in nature, severity, or frequency (i.e. not described in study-related documents)
- Related or possibly related to participation in the research (i.e. there is a reasonable possibility that the research procedures may have caused the incident experience, or outcome)
- Places subjects or others at greater risk of physical, psychological, economic, or social harm

5.2 Recording and Reporting Adverse Events

Because this is not an interventional study, AE recording and reporting will be limited to AEs that may be related to study procedures (i.e., blood draws). All AEs that occur from the time that subjects sign informed consent through their completion of the research blood draws will be collected and reported.

Study staff must actively seek information about adverse events and unanticipated problems involving risk to subjects or others. Adverse events must be recorded in the case report form (CRF).

The clinical course of each AE must be followed until resolution, stabilization, or the investigator's determination that study participation did not cause the AE. SAEs that are ongoing at the end of the study must be followed through final outcome. Any SAE that occurs after a research blood draw and is considered to be possibly related to the study should be recorded and reported immediately.

All SAEs must be reported to Cognizance Biomarkers as soon as possible, but no later 24 hours after the study staff becomes aware of the event. The event should be reported using Cognizance Biomarkers' SAE report form, and include all readily available information. Follow-up reports must be issued as new

information about the SAE becomes available. Additionally, study staff must follow institutional requirements for reporting AEs, SAEs, and unanticipated problems involving risks to subjects and others.

5.3 Medical Monitoring

Cognizance Biomarkers will monitor the study progress on-site at periodic intervals throughout the study. This will include careful assessment and appropriate reporting of adverse events as noted above.

6 Data Handling

6.1 Research Subjects

Source documentation of subjects' medical history, clinical epilepsy diagnostic procedures, and epilepsy medications will remain in subjects' medical charts. Signed Informed Consent/ HIPPA forms will be stored in separate study binders. The only study procedures associated with this research are blood draws; all other data used for the study will be extracted from subjects' medical records and recorded on the CRFs.

6.2 Control Subjects

Data will be limited to the following, and will be self-reported at the time of Consent:

- Gender
- Age
- Confirmation that they do not experience seizure or suspected seizures
- Confirmation that they are not being treated with anti-seizure medications for any condition

All data will be stored in study binders, separate from signed Informed Consent/ HIPPA forms.

7 Data Analysis

7.1 Sample Size Determination

Enrollment will continue until 240 outpatients have joined the study, provided a single blood sample, and undergone an EEG that is accessible to the study team. Subjects who either do not provide a blood sample or do not undergo an EEG will be replaced.

This sample size is based on the need for 68 subjects per group and reflects the expectation that within six months of initial presentation, 50% will be diagnosed with epilepsy, 30% will be diagnosed with non-epileptic events, and 20% will have an unknown diagnosis.

Control samples will be used as needed to further validate the CARPE test.

7.2 Statistical Methods

The goals of the study are to determine whether the CARPE value can be used effectively and accurately to diagnose patients with epilepsy, and to establish the threshold for diagnosis. The CARPE AUC from

the ROC curve of our pilot data was 1.0 and the sICAM5 AUC was 0.87. For a conservative estimate, the sample size will be estimated based on the sICAM5 AUC, which is the lower value.

Accuracy: To determine the accuracy of the test, we will construct ROC curves for sensitivity and specificity of CARPE by varying the threshold value for classifying epilepsy patients. The gold standard for constructing the ROC curve will be the diagnosis as determined by the panel of three independent epileptologists. The aim of this part of the study is to construct a reliable ROC curve. It is likely that the gold standard panel will not come to a conclusive diagnosis approximately 20% of the time, and these unknowns will not be included in the ROC determination. We will consider a conclusive diagnosis to be agreement between at least two of the three epileptologists that the subject is epileptic or non-epileptic at the time of evaluation. We use the AUC of the ROC curve as a global measure of the effectiveness of these measurements. To this end, we assume that the prevalence rate is 50% for epilepsy among all the study subjects. Moreover, we assume that the CARPE and sICAM5 measures are normally distributed for both epilepsy and non-epilepsy subjects.

With an Alpha of 0.05 and a Beta of 0.2, comparing the AUC of 0.87 to the null hypothesis value of 0.7, the minimal required sample size per group is 68. The smaller group in this study will be subjects who have the diagnosis of non-epileptic attacks, which will likely represent 30% of the whole group. This justifies the sample size n=240 as adequate for the study. The determination of success will be based on this analysis.

Threshold: The second analysis will establish the threshold values to be used for the expected subsequent study. The goal is to choose the threshold value of CARPE for identifying epilepsy subjects with specificity of 0.8 and sensitivity of 0.8. For this analysis the entire study group will be analyzed. The panel of epileptologists will provide their best estimate of likely epileptic or likely non-epileptic events for this analysis (agreement among two members of the panel will be sufficient).

Our gold standard comparator in for this study is the panel of three independent epileptologists rather than EEG alone due to the fact that EEG's sensitivity for detecting epilepsy is only 50%.[9,10] It is understood that CARPE must significantly exceed this in order to be considered a useful diagnostic test.

8 Sample Collection

- Outpatient Research and Control Subjects will be asked to provide up to two 5ml tubes of blood via venipuncture for research at enrollment. It will not be considered a violation if the Control Subject gives less than two tubes of blood (i.e., if he or she gives one tube or less, or if the phlebotomist is unable to obtain any amount of blood).
- Subjects who undergo cVEEG monitoring at HUP will be asked to provide no more than two 5ml sample of blood via venipuncture per day (12am-11:59pm) for research.
- All Research Subjects will have the option of providing an additional single 5ml sample of blood via venipuncture for research at any regularly scheduled clinic visit that takes place within 12 months of enrollment (Control Subjects will only be asked to give blood at the time of enrollment.)

All samples will be collected at the Hospital of the University of Pennsylvania's Department of Neurology.

All blood draws are optional, and it will not be considered a protocol deviation of a blood draw is missed for any reason (including subject preference).

9 Sample Processing and Storage

9.1 Duration of Storage

Cognizance Biomarkers will store subjects' samples for approximately five years; this will allow ample time for batch processing and re-testing as necessary. We will destroy all samples that have not been processed after five years.

9.2 Vial Labeling

All samples collected for research will be de-identified and labeled with the date, protocol number, and a numerical or alphanumerical code. The samples will not include any subject identifiers.

9.3 Sample Preparation

As soon as possible after it is drawn, plasma will be isolated from whole blood and stored at -80^{o}C. The plasma will be assayed using academic or commercial testing.

10 Privacy and Confidentiality

10.1 Study Samples

Only personnel authorized by the University of Pennsylvania's IRB and Cognizance Biomarkers will have direct access to the study samples and documents. Samples will be labeled with only the following information: date of collection, protocol number, and numerical or alphanumerical ID. Control Samples will be designated as such with the letter "C." Any subsequent vials used during research will also be free from personally identifying information and may only include, at most, the information listed above.

10.2 Medical Information

All records pertaining to the identity of participants in the Research Repository will be maintained as private and confidential. Data will be labeled with the Subject ID (001, 002, etc) and protocol number only.

11 References

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