Sample Application for Small Business Funding

Through the Small Business Innovation Research (SBIR) and Small Business Technology Transfer (STTR) programs, NIA aims to help small businesses develop effective treatments and interventions for healthy aging. NIH small business funding is competitive, and resubmissions are a common and important part of the award process.

Find more NIA sample applications and information about SBIR/STTR funding:
https://www.nia.nih.gov/research/sbir/nia-small-business-sample-applications

PI: **Vollrath, Ben**	Title: Blood-based diagnostics for Alzheimer's Disease	
Received: 04/07/2014	FOA: PA14-072	Council: 10/2014
Competition ID: ADOBE-FORMS-B2	FOA Title: PHS 2014-02 OMNIBUS SOLICITATION OF THE NIH FOR SMALL BUSINESS TECHNOLOGY TRANSFER GRANT APPLICATIONS (PARENT STTR [R41/R42]	
1 R42 AG049562-01	Dual:	Accession Number: 3686625
IPF: 10029662	Organization: AMPRION, INC.	
Former Number:	Department:	
IRG/SRG: ZRG1 ETTN-M (11)B	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: [redacted] Year 2: [redacted] Year 3: [redacted]	Animals: Y Humans: Y Clinical Trial: N Current HS Code: E4 HESC: N	New Investigator: Early Stage Investigator:
Senior/Key Personnel:	*Organization:*	*Role Category:*
Benedikt Vollrath	AMPRION INC	PD/PI
Claudio Soto	The University of Texas Health Science Center	MPI

OMB Number: 4040-0001
Expiration Date: 06/30/2011

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. * TYPE OF SUBMISSION
☐ Pre-application ☒ Application ☐ Changed/Corrected Application

2. DATE SUBMITTED | **Applicant Identifier**

4. a. Federal Identifier

b. Agency Routing Identifier

5. APPLICANT INFORMATION *** Organizational DUNS:** [redacted]

* Legal Name: AMPRION INC

Department: | Division:

* Street1: [redacted]

Street2:

* City: Houston | County / Parish: [redacted]

* State: TX: Texas | Province:

* Country: USA: UNITED STATES | * ZIP / Postal Code: [redacted]

Person to be contacted on matters involving this application

Prefix: Dr. | * First Name: Benedikt | Middle Name:

* Last Name: Vollrath | Suffix:

* Phone Number: [redacted] | Fax Number:

Email: [redacted]

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN):* [redacted]

7. * TYPE OF APPLICANT: R: Small Business

Other (Specify):

Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:
☒ New ☐ Resubmission
☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).
☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration
☐ E. Other (specify):

* Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. * NAME OF FEDERAL AGENCY: National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:
TITLE:

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:
Blood-based diagnostics for Alzheimer's Disease

12. PROPOSED PROJECT:
* Start Date: 09/01/2014 | * Ending Date: 08/31/2017

*** 13. CONGRESSIONAL DISTRICT OF APPLICANT** TX-07

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. | * First Name: Benedikt | Middle Name:

* Last Name: Vollrath | Suffix:

Position/Title: Chief Operating Officer

* Organization Name: AMPRION INC

Department: | Division:

* Street1: [redacted]

Street2:

* City: Houston | County / Parish: [redacted]

* State: TX: Texas | Province:

* Country: USA: UNITED STATES | * ZIP / Postal Code: [redacted]

* Phone Number: [redacted] | Fax Number:

* Email: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested [redacted]

b. Total Non-Federal Funds [redacted]

c. Total Federal & Non-Federal Funds [redacted]

d. Estimated Program Income [redacted]

16. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☐ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☒ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious. or fraudulent statements or claims may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 18, Section 1001)

☒ *** I agree**

**** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.***

18. SFLLL or other Explanatory Documentation

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: [redacted] * First Name: [redacted] Middle Name:

* Last Name: [redacted] Suffix:

* Position/Title: Chief Executive Officer

* Organization: AMPRION INC

Department: Division:

* Street1: [redacted]

Street2:

* City: Houston County / Parish: [redacted]

* State: TX: Texas Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: [redacted]

* Phone Number: [redacted] Fax Number:

* Email: [redacted]

*** Signature of Authorized Representative**

Ben Vollrath

*** Date Signed**

04/07/2014

20. Pre-application

Add Attachment | Delete Attachment | View Attachment

OMB Number: 4040-0010
Expiration Date: 08/31/2011

Project/Performance Site Location(s)

Project/Performance Site Primary Location ☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: Amprion Inc

DUNS Number: [redacted]

* Street1: [redacted]

Street2:

* City: Houston County: [redacted]

* State: TX: Texas

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: [redacted] * Project/ Performance Site Congressional District: TX-007

Project/Performance Site Location 1 ☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: University of Texas Health Science Center at Houston

DUNS Number: [redacted]

* Street1: [redacted]

Street2:

* City: Houston County: [redacted]

* State: TX: Texas

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: [redacted] * Project/ Performance Site Congressional District: [redacted]

Additional Location(s) Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Other Project Information

OMB Number: 4040-0001
Expiration Date: 6/30/2016

1. Are Human Subjects Involved? ☒ Yes ☐ No

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☒ Yes ☐ No

If yes, check appropriate exemption number. ☐ 1 ☐ 2 ☐ 3 ☒ 4 ☐ 5 ☐ 6

If no, is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Human Subject Assurance Number:

2. Are Vertebrate Animals Used? ☒ Yes ☐ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☒ Yes ☐ No

IACUC Approval Date:

Animal Welfare Assurance Number: A3413-01

3. Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. Does this Project Have an Actual or Potential Impact - positive or negative - on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place? ☐ Yes ☒ No

5.a. If yes, please explain:

6. Does this project involve activities outside of the United States or partnerships with international collaborators? ☐ Yes ☒ No

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. Project Summary/Abstract 1237-ABSTRACT.pdf Add Attachment | Delete Attachment | View Attachment

8. Project Narrative 1238-NARRATIVE.pdf Add Attachment | Delete Attachment | View Attachment

9. Bibliography & References Cited 1239-Literature cited.pdf Add Attachment | Delete Attachment | View Attachment

10. Facilities & Other Resources 1240-RESOURCES-UTH.pdf Add Attachment | Delete Attachment | View Attachment

11. Equipment 1241-MAJOR EQUIPMENT.pdf Add Attachment | Delete Attachment | View Attachment

12. Other Attachments Add Attachments | Delete Attachments | View Attachments ☐

ABSTRACT

This proposal is for a phase I/II fast track project for the STTR program with the main goal to develop a blood test for Alzheimer's disease (AD) diagnosis. AD is the most common dementia in the elderly population and one of the leading causes of death in the developed world. One of the main problems in AD is the lack of an early, sensitive and objective laboratory diagnosis to identify individuals that will develop the disease before substantial brain damage. Compelling evidences point that the hallmark event in AD is the misfolding, aggregation and brain accumulation of amyloid-beta (Aβ) protein. Aβ aggregation follows a seeding-nucleation mechanism and involves several intermediates, including soluble oligomers and protofibrils. Recent evidence has shown that Aβ oligomers are circulating in biological fluids and these structures appear to be key for inducing brain degeneration in AD. Our working hypothesis is that detection of misfolded Aβ oligomers circulating in blood may be the basis for an early biochemical diagnosis for AD. Our approach is to use the functional property of misfolded oligomers of being capable to catalyze the polymerization of the monomeric protein as a way to detect them. We have recently invented the protein misfolding cyclic amplification (PMCA), which represent a platform technology to detect very small quantities of seeding-competent misfolded oligomeric proteins associated with various protein misfolding diseases. Currently, PMCA has been adapted to detect misfolded prion protein implicated in prion diseases in various biological fluids, including blood and urine and more recently soluble Aβ oligomers in cerebrospinal fluid of AD patients. The major goal of this project is to adapt the PMCA technology for specific and highly sensitive detection of misfolded Aβ oligomers in human blood, perform studies of specificity and sensitivity using large number of samples and evaluate the utility of Aβ-PMCA for pre-clinical identification of people in the way to develop AD. The results generated in this project may lead to the first biochemical test for blood-based diagnosis of AD. The studies included in this project will constitute the basis for regulatory approval of the test that Amprion will commercialize.

NARRATIVE

Development of a blood-based biochemical assay for the sensitive, early and non-invasive diagnosis of Alzheimer's disease is a top medical priority, essential to permit efficient treatment of this devastating disease. This project proposes to develop the protein misfolding cyclic amplification (PMCA) technology to detect with high sensitivity and specificity amyloid-beta oligomers which are considered the key molecules responsible for neurodegeneration in AD. In this project we have put together the relevant technical and business expertise and secured the availability to key samples to permit the successful development, validation and approval of the test.

RESOURCES AT UTH

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Located on the 4 floor of the new Medical School extension Research building, Dr Soto's lab have more than 4500 square feet lab space divided in 10 rooms: A large room dedicated to biochemical and general lab procedures, a large BSL2 room dedicated to work with rodent prion infectious material, a BSL3 room to work with prions of human and cervid origin, a cell culture room, a molecular biology and bacteriology room, an animal surgery room, a histology room, an animal behavioral room, a room for microscopic and image analysis equipment and a cold room.

Clinical:

Through physicians associated to the Mitchell Center (Dr Paul Shulz's group) we have state-of-the-art facilities for clinical care and clinical research, including collection of tissues and biological fluids from patients affected by diverse neurodegenerative diseases

Animal:

Facilities available at the University of Texas Medical School will be used to house the animals and to perform survival surgery. We have dedicated facilities to maintain our multiple animal colonies and to carry out breeding, animal genotyping and sample collection.

Computer:

The laboratory is equipped with 20 personal computers, several printers and network access to online services.

Office:

We have six offices. One is used by Dr. C. Soto, another is a shared office for 4 postdoctoral fellows, a secretarial office, two offices for junior faculty [redacted] and a seminar room equipped with video-conference.

Other:

The University offers access to several Core facilities, such as protein production, purification, mass spectroscopy, optical imaging, transgenic production, molecular biology, in vivo imaging, NMR, bio-informatics, high throughput screening, bio-statistics, etc.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Dr Soto's laboratory is fully equipped for protein biochemistry, cell biology, histology, molecular biology, drug screening, in vivo biology, animal behavior and work with infectious prion material. Available are full cell and tissue culture facilities, several centrifuges, HPLC, confocal, fluorescence and electron microscopy, PCR machines, several automated microsonicators, spectrophotometers, ELISA plate reader, freezers, electrophoresis and blotting apparatus, several optical microscopes, image analysis system, stereotaxic instruments, histology equipment (cryostat, microtome, vibratome), autoclave, spectrofluorometer, Fourier-transformed infrared spectroscopy, radioactivity counters, electrophysiological equipment, and many small general lab equipments. Through collaboration with our scientists in our School we have access to use circular dichroism, atomic force microscopy, cryoelectron microscopy, etc.

OMB Number: 4040-0001
Expiration Date: 06/30/2011

Previous Period

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: AMPRION INC

Delete Entry *** Start Date:** 09/01/2014 *** End Date:** 08/31/2015 **Budget Period** 1

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Dr.	Benedikt		Vollrath		PD/PI	[redacted]	[redacted]			[redacted]	[redacted]	[redacted]
2.													
3.													
4.													
5.													
6.													
7.													
8.													

9. **Total Funds requested for all Senior Key Persons in the attached file**

Total Senior/Key Person [redacted]

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
[redacted]	Research Assistant	6.00			[redacted]	[redacted]	[redacted]
[redacted]	**Total Number Other Personnel**					**Total Other Personnel**	[redacted]

Total Salary, Wages and Fringe Benefits (A+B) [redacted]

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 1

* ORGANIZATIONAL DUNS: [redacted]

* Budget Type: ☒ Project ☐ Subaward/Consortium

Enter name of Organization: AMPRION INC

Delete Entry * Start Date: 09/01/2014 * End Date: 08/31/2015 Budget Period 1

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

	Equipment item	* Funds Requested ($)
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
11.	Total funds requested for all equipment listed in the attached file	
	Total Equipment	

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

D. Travel — **Funds Requested ($)**

1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	[redacted]
2.	Foreign Travel Costs	
	Total Travel Cost	[redacted]

E. Participant/Trainee Support Costs — **Funds Requested ($)**

1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other	
	Number of Participants/Trainees	Total Participant/Trainee Support Costs

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION F-K, BUDGET PERIOD 1

Next Period

* ORGANIZATIONAL DUNS: [redacted]

* Budget Type: ☒ Project ☐ Subaward/Consortium

Enter name of Organization: AMPRION INC

Delete Entry * Start Date: 09/01/2014 * End Date: 08/31/2015 Budget Period 1

F. Other Direct Costs	Funds Requested ($)
1. Materials and Supplies	[redacted]
2. Publication Costs	
3. Consultant Services	[redacted]
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	[redacted]
6. Equipment or Facility Rental/User Fees	[redacted]
7. Alterations and Renovations	
8. Other Costs	[redacted]
9.	
10.	
Total Other Direct Costs	[redacted]

G. Direct Costs	Funds Requested ($)
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Indirect cost	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				
	Total Indirect Costs			[redacted]

Cognizant Federal Agency

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)
	[redacted]

K. * Budget Justification 1250-Budget Justification.pdf Add Attachment Delete Attachment View Attachment

(Only attach one file.)

OMB Number: 4040-0001
Expiration Date: 06/30/2011

Previous Period

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 2

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: AMPRION INC

Delete Entry *** Start Date:** 09/01/2015 *** End Date:** 08/31/2016 **Budget Period** 2

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Dr.	Benedikt		Vollrath		PD/PI	[redacted]	[redacted]			[redacted]	[redacted]	[redacted]
2.													
3.													
4.													
5.													
6.													
7.													
8.													

9. **Total Funds requested for all Senior Key Persons in the attached file**

Total Senior/Key Person [redacted]

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
[redacted]	Research Assistant	[redacted]			[redacted]	[redacted]	[redacted]
[redacted]	**Total Number Other Personnel**					**Total Other Personnel**	[redacted]

Total Salary, Wages and Fringe Benefits (A+B) [redacted]

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 2

* ORGANIZATIONAL DUNS: [redacted]

* Budget Type: ☒ Project ☐ [redacted]

Enter name of Organization: AMPRION INC

Delete Entry * Start Date: 09/01/2015 * End Date: 08/31/2016 Budget Period 2

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

	Equipment item	* Funds Requested ($)
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
11.	Total funds requested for all equipment listed in the attached file	
	Total Equipment	

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

D. Travel — **Funds Requested ($)**

1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	[redacted]
2.	Foreign Travel Costs	
	Total Travel Cost	[redacted]

E. Participant/Trainee Support Costs — **Funds Requested ($)**

1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other	
	Number of Participants/Trainees	Total Participant/Trainee Support Costs

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION F-K, BUDGET PERIOD 2

Next Period

* ORGANIZATIONAL DUNS: [redacted]

* Budget Type: ☒ Project ☐ Subaward/Consortium

Enter name of Organization: AMPRION INC

Delete Entry Start Date: 09/01/2015 * End Date: 08/31/2016 Budget Period 2

F. Other Direct Costs	Funds Requested ($)
1. Materials and Supplies	[redacted]
2. Publication Costs	
3. Consultant Services	[redacted]
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	[redacted]
6. Equipment or Facility Rental/User Fees	[redacted]
7. Alterations and Renovations	
8. Other	[redacted]
9.	
10.	
Total Other Direct Costs	[redacted]

G. Direct Costs	Funds Requested ($)
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	[redacted]	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				
	Total Indirect Costs			[redacted]

Cognizant Federal Agency

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)
	[redacted]

K. * Budget Justification 1250-Budget Justification.pdf Add Attachment Delete Attachment View Attachment

(Only attach one file.)

OMB Number: 4040-0001
Expiration Date: 06/30/2011

Previous Period

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 3

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ☒ Project ☐ Subaward/Consortium

Enter name of Organization: AMPRION INC

Delete Entry *** Start Date:** 09/01/2016 *** End Date:** 08/31/2017 **Budget Period** 3

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Dr.	Benedikt		Vollrath		PD/PI	[redacted]	[redacted]			[redacted]	[redacted]	[redacted]
2.													
3.													
4.													
5.													
6.													
7.													
8.													

9. **Total Funds requested for all Senior Key Persons in the attached file**

Total Senior/Key Person [redacted]

Additional Senior Key Persons: Add Attachment Delete Attachment View Attachment

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
[redacted]	Research Assistant	[redacted]			[redacted]	[redacted]	[redacted]
[redacted]	**Total Number Other Personnel**					**Total Other Personnel**	[redacted]

Total Salary, Wages and Fringe Benefits (A+B) [redacted]

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 3

* ORGANIZATIONAL DUNS: [redacted]

* Budget Type: ☒ Project ☐ Subaward/Consortium

Enter name of Organization: AMPRION INC

Delete Entry * Start Date: 09/01/2016 * End Date: 08/31/2017 Budget Period 3

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

	Equipment item	* Funds Requested ($)
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
11.	Total funds requested for all equipment listed in the attached file	
	Total Equipment	

Additional Equipment: Add Attachment Delete Attachment View Attachment

D. Travel **Funds Requested ($)**

1.	Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	[redacted]
2.	Foreign Travel Costs	
	Total Travel Cost	[redacted]

E. Participant/Trainee Support Costs **Funds Requested ($)**

1.	Tuition/Fees/Health Insurance	
2.	Stipends	
3.	Travel	
4.	Subsistence	
5.	Other	
	Number of Participants/Trainees	Total Participant/Trainee Support Costs

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION F-K, BUDGET PERIOD 3

Next Period

* ORGANIZATIONAL DUNS: [redacted]

* Budget Type: ☒ Project ☐ Subaward/Consortium

Enter name of Organization: AMPRION INC

Delete Entry * Start Date: 09/01/2016 * End Date: 08/31/2017 Budget Period 3

F. Other Direct Costs	Funds Requested ($)
1. Materials and Supplies	[redacted]
2. Publication Costs	
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	[redacted]
6. Equipment or Facility Rental/User Fees	[redacted]
7. Alterations and Renovations	
8. Other costs	[redacted]
9.	
10.	
Total Other Direct Costs	[redacted]

G. Direct Costs	Funds Requested ($)
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	[redacted]	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				
	Total Indirect Costs			[redacted]

Cognizant Federal Agency

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)
	[redacted]

K. * Budget Justification 1250-Budget Justification.pdf Add Attachment Delete Attachment View Attachment

(Only attach one file.)

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)
Section A, Senior/Key Person	[redacted]
Section B, Other Personnel	[redacted]
Total Number Other Personnel	[redacted]
Total Salary, Wages and Fringe Benefits (A+B)	[redacted]
Section C, Equipment	
Section D, Travel	[redacted]
1. Domestic	[redacted]
2. Foreign	
Section E, Participant/Trainee Support Costs	
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
6. Number of Participants/Trainees	
Section F, Other Direct Costs	[redacted]
1. Materials and Supplies	[redacted]
2. Publication Costs	
3. Consultant Services	[redacted]
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	[redacted]
6. Equipment or Facility Rental/User Fees	[redacted]
7. Alterations and Renovations	
8. Other 1	[redacted]
9. Other 2	
10. Other 3	
Section G, Direct Costs (A thru F)	[redacted]
Section H, Indirect Costs	[redacted]
Section I, Total Direct and Indirect Costs (G + H)	[redacted]
Section J, Fee	[redacted]

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 8007715940000

*** Budget Type:** ○Project ●Subaward/Consortium

Enter name of Organization: The University of Houston Health Science Center at Houston

*** Start Date:** 09-01-2014 *** End Date:** 08-31-2015 **Budget Period: 1**

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.		Claudio		Soto	PhD	PD/PI	[redacted]	[redacted]			[redacted]	[redacted]	[redacted]

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name: Mime Type: **Total Senior/Key Person** [redacted]

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits	* Funds Requested ($)
[redacted]	Post Doctoral Associates	[redacted]			[redacted]	[redacted]	[redacted]
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
[redacted]	**Total Number Other Personnel**					**Total Other Personnel**	[redacted]
						Total Salary, Wages and Fringe Benefits (A+B)	[redacted]

RESEARCH & RELATED Budget {A-B} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ○Project ●Subaward/Consortium

Enter name of Organization: The University of Houston Health Science Center at Houston

*** Start Date:** 09-01-2014 *** End Date:** 08-31-2015 **Budget Period: 1**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	* Funds Requested ($)

Total funds requested for all equipment listed in the attached file

Total Equipment

Additional Equipment: File Name: Mime Type:

D. Travel **Funds Requested ($)**

1. [redacted]

Total Travel Cost

E. Participant/Trainee Support Costs **Funds Requested ($)**

1. [redacted]

Number of Participants/Trainees **Total Participant/Trainee Support Costs**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ○Project ●Subaward/Consortium

Enter name of Organization: The University of Houston Health Science Center at Houston

*** Start Date:** 09-01-2014 *** End Date:** 08-31-2015 **Budget Period: 1**

F. Other Direct Costs	**Funds Requested ($)**
1. Materials and Supplies	[redacted]
2. Publication Costs	
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
Total Other Direct Costs	[redacted]

G. Direct Costs	**Funds Requested ($)**
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

Indirect Cost Type	**Indirect Cost Rate (%)**	**Indirect Cost Base ($)**	*** Funds Requested ($)**
1. [redacted]	[redacted]	[redacted]	[redacted]
		Total Indirect Costs	[redacted]

Cognizant Federal Agency [redacted]

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	**Funds Requested ($)**
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	**Funds Requested ($)**

K. * Budget Justification File Name: 1234-budget justification-UT.pdf Mime Type: application/pdf

(Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 2

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ○Project ●Subaward/Consortium

Enter name of Organization: The University of Houston Health Science Center at Houston

*** Start Date:** 09-01-2015 *** End Date:** 08-31-2016 **Budget Period: 2**

A. Senior/Key Person

Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Claudio		Soto	PhD	PD/PI		[redacted]			[redacted]	[redacted]	[redacted]

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name: Mime Type: **Total Senior/Key Person** [redacted]

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits	* Funds Requested ($)
[redacted]	Post Doctoral Associates	[redacted]			[redacted]	[redacted]	[redacted]
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
[redacted]	Research Assistant	[redacted]			[redacted]	[redacted]	[redacted]
[redacted]	**Total Number Other Personnel**					**Total Other Personne**	[redacted]
						Total Salary, Wages and Fringe Benefits (A+B)	[redacted]

RESEARCH & RELATED Budget {A-B} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 2

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ○Project ●Subaward/Consortium

Enter name of Organization: The University of Houston Health Science Center at Houston

*** Start Date:** 09-01-2015 *** End Date:** 08-31-2016 **Budget Period: 2**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	* Funds Requested ($)
1. ELISA plate reader	[redacted]

Total funds requested for all equipment listed in the attached file

Total Equipment [redacted]

Additional Equipment: File Name: Mime Type:

D. Travel	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	[redacted]
2. Foreign Travel Costs	[redacted]
Total Travel Cost	[redacted]

E. Participant/Trainee Support Costs	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other:	

Number of Participants/Trainees **Total Participant/Trainee Support Costs**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, BUDGET PERIOD 2

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ○Project ●Subaward/Consortium

Enter name of Organization: The University of Houston Health Science Center at Houston

*** Start Date:** 09-01-2015 *** End Date:** 08-31-2016 **Budget Period: 2**

F. Other Direct Costs	Funds Requested ($)
1. Materials and Supplies	[redacted]
2. Publication Costs	[redacted]
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8. Maintenance and core facilities costs	[redacted]
Total Other Direct Costs	[redacted]

G. Direct Costs	Funds Requested ($)
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1. [redacted]	[redacted]	[redacted]	[redacted]
		Total Indirect Costs	[redacted]

Cognizant Federal Agency [redacted]
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)

K. * Budget Justification File Name: 1234-budget justification-UT.pdf Mime Type: application/pdf
(Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 3

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ○Project ●Subaward/Consortium

Enter name of Organization: The University of Houston Health Science Center at Houston

*** Start Date:** 09-01-2016 *** End Date:** 08-31-2017 **Budget Period: 3**

A. Senior/Key Person

Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.	Claudio		Soto	PhD	PD/PI		[redacted]			[redacted]	[redacted]	[redacted]

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name: Mime Type: **Total Senior/Key Person** [redacted]

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits	* Funds Requested ($)
[redacted]	Post Doctoral Associates	[redacted]			[redacted]	[redacted]	[redacted]
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
[redacted]	Research Assistant	[redacted]			[redacted]	[redacted]	[redacted]
[redacted]	**Total Number Other Personnel**					**Total Other Personne**	[redacted]
						Total Salary, Wages and Fringe Benefits (A+B)	[redacted]

RESEARCH & RELATED Budget {A-B} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 3

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ○Project ●Subaward/Consortium

Enter name of Organization: The University of Houston Health Science Center at Houston

*** Start Date:** 09-01-2016 *** End Date:** 08-31-2017 **Budget Period: 3**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	* Funds Requested ($)

Total funds requested for all equipment listed in the attached file

Total Equipment

Additional Equipment: File Name: Mime Type:

D. Travel	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	[redacted]
2. Foreign Travel Costs	[redacted]
Total Travel Cost	[redacted]

E. Participant/Trainee Support Costs	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other:	

Number of Participants/Trainees **Total Participant/Trainee Support Costs**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, BUDGET PERIOD 3

*** ORGANIZATIONAL DUNS:** [redacted]

*** Budget Type:** ○Project ●Subaward/Consortium

Enter name of Organization: The University of Houston Health Science Center at Houston

*** Start Date:** 09-01-2016 *** End Date:** 08-31-2017 **Budget Period: 3**

F. Other Direct Costs	Funds Requested ($)
1. Materials and Supplies	[redacted]
2. Publication Costs	[redacted]
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8. Maintenance and core facilities costs	[redacted]
Total Other Direct Costs	[redacted]

G. Direct Costs	Funds Requested ($)
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1. [redacted]	[redacted]	[redacted]	[redacted]
		Total Indirect Costs	[redacted]

Cognizant Federal Agency

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)

K. * Budget Justification	File Name: 1234-budget justification-UT.pdf	Mime Type: application/pdf
	(Only attach one file.)	

RESEARCH & RELATED Budget {F-K} (Funds Requested)

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)
Section A, Senior/Key Person	[redacted]
Section B, Other Personnel	[redacted]
Total Number Other Personnel	5
Total Salary, Wages and Fringe Benefits (A+B)	[redacted]
Section C, Equipment	[redacted]
Section D, Travel	[redacted]
1. Domestic	[redacted]
2. Foreign	[redacted]
Section E, Participant/Trainee Support Costs	
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
6. Number of Participants/Trainees	
Section F, Other Direct Costs	[redacted]
1. Materials and Supplies	[redacted]
2. Publication Costs	[redacted]
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8. Other 1	[redacted]
9. Other 2	
10. Other 3	
Section G, Direct Costs (A thru F)	[redacted]
Section H, Indirect Costs	[redacted]
Section I, Total Direct and Indirect Costs (G + H)	[redacted]
Section J, Fee	

SBIR/STTR Information

OMB Number: 4040-0001
Expiration Date: 6/30/2016

*** Program Type (select only one)**

☐ SBIR ☒ STTR

☐ Both *(See agency-specific instructions to determine whether a particular agency allows a single submission for both SBIR and STTR)*

*** SBIR/STTR Type (select only one)**

☐ Phase I ☐ Phase II

☒ Fast-Track *(See agency-specific instructions to determine whether a particular agency participates in Fast-Track)*

Questions 1-7 must be completed by all SBIR and STTR Applicants:

☒ Yes ☐ No	* 1a. Do you certify that at the time of award your organization will meet the eligibility criteria for a small business as defined in the funding opportunity announcement?
	* 1b. Anticipated Number of personnel to be employed at your organization at the time of award. 2
☐ Yes ☒ No	* 2. Does this application include subcontracts with Federal laboratories or any other Federal Government agencies? * If yes, insert the names of the Federal laboratories/agencies:
☐ Yes ☒ No	* 3. Are you located in a HUBZone? To find out if your business is in a HUBZone, use the mapping utility provided by the Small Business Administration at its web site: http://www.sba.gov
☒ Yes ☐ No	* 4. Will all research and development on the project be performed in its entirety in the United States? If no, provide an explanation in an attached file. * Explanation: Add Attachment | Delete Attachment | View Attachment
☐ Yes ☒ No	* 5. Has the applicant and/or Program Director/Principal Investigator submitted proposals for essentially equivalent work under other Federal program solicitations or received other Federal awards for essentially equivalent work? * If yes, insert the names of the other Federal agencies:
☒ Yes ☐ No	* 6. Disclosure Permission Statement: If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?
	* 7. Commercialization Plan: If you are submitting a Phase II or Phase I/Phase II Fast-Track Application, include a Commercialization Plan in accordance with the agency announcement and/or agency-specific instructions. * Attach File: 1249-Commercialization Plan Amp Add Attachment | Delete Attachment | View Attachment

SBIR/STTR Information

SBIR-Specific Questions:

Questions 8 and 9 apply only to SBIR applications. If you are submitting ONLY an STTR application, leave questions 8 and 9 blank and proceed to question 10.

☐ Yes ☐ No	* 8. Have you received SBIR Phase II awards from the Federal Government? If yes, provide a company commercialization history in accordance with agency-specific instructions using this attachment. * Attach File: [ ] Add Attachment | Delete Attachment | View Attachment
☐ Yes ☐ No	* 9. Will the Project Director/Principal Investigator have his/her primary employment with the small business at the time of award?

STTR-Specific Questions:

Questions 10 and 11 apply only to STTR applications. If you are submitting ONLY an SBIR application, leave questions 10 and 11 blank.

☒ Yes ☐ No	* 10. Please indicate whether the answer to BOTH of the following questions is TRUE: (1) Does the Project Director/Principal Investigator have a formal appointment or commitment either with the small business directly (as an employee or a contractor) OR as an employee of the Research Institution, which in turn has made a commitment to the small business through the STTR application process; AND (2) Will the Project Director/Principal Investigator devote at least 10% effort to the proposed project?
☒ Yes ☐ No	* 11. In the joint research and development proposed in this project, does the small business perform at least 40% of the work and the research institution named in the application perform at least 30% of the work?

COMMERCIALIZATION PLAN

I. VALUE OF THE STTR PROJECT, EXPECTED OUTCOMES, AND IMPACT
II.

The major goal of this STTR proposal is to seek funding for developing a highly sensitive and specific blood-based diagnostic for Alzheimer's disease (AD). AD is a progressive neurodegenerative disorder characterized by cognitive and memory defects and progressive impairment of daily activities. Current estimates suggest that approximately 5% of the population aged 65 and over, and approximately 20% of the population aged 80 years and over are affected by the disease. It is also estimated that worldwide more than 115 million people will be affected by dementia by 2050, the majority of whom will be AD.

Despite this staggering disease burden, and despite the fact that the biotechnology and pharmaceutical industry have invested significant resources in developing novel treatments for AD, the field is littered with late-stage clinical failures, and so far disease-modifying treatments remain elusive.

In contrast to many other disorders of the Central Nervous System, such as depression, schizophrenia or bipolar disorder, a well-supported hypothesis for the pathogenesis of AD has been formulated that can be used to develop disease-modifying therapeutics. While formal proof of this hypothesis requires positive outcomes in clinical trials with a novel therapeutic targeting the proposed disease mechanism, this hypothesis has strong data support from preclinical studies, clinical observations in patients as well as human genetics. Amyloid plaques, formed by aggregation of amyloid-β peptides, have long been regarded as the hallmark of the disease, and as a critical component of pathogenesis. However, plaque load in patients correlates only poorly with clinical scores or disease progression, questioning the direct link between amyloid-β plaques and AD pathogenesis. A large body of data now supports the role of soluble amyloid-β oligomers - smaller aggregates of amyloid-β that ultimately form fibrillary plaques - as the critical pathogenic species in AD. Soluble amyloid-β oligomers disrupt synaptic integrity at very low concentrations, and chronic exposure to soluble amyloid-β oligomers leads to tau hyperphosphorylation, neurofibrillary tangle formation and neurodegeneration, all critical hallmarks of AD pathology. Crucially, levels of soluble amyloid-β oligomers measured in the Cerebro-spinal Fluid (CSF) correlate well with disease scores in AD, further supporting the hypothesis that soluble amyloid-β oligomers are the critical pathogenic molecular species in AD.

Current diagnosis of AD is primarily performed using cognitive tests such as the Mini-mental State Evaluation (MMSE) or the ADAS-Cog scale. In addition, CSF analysis is often performed analyzing amyloid-beta peptides, tau or phosphorylated tau protein as read-outs. The challenge in using these measures is that patients will have to exhibit

substantial synaptic defects at the cellular level long before cognitive measures or currently used biomarkers start to indicate a pathology, and the disease is therefore diagnosed very late in the overall course. Developing drugs that are capable of reversing disease, even just stopping disease progression, is inherently very difficult or even impossible at this stage, and recent trail failures of drug candidates on Phase III of clinical testing support this contention. The FDA has issued recent guidance supporting the development and use of technologies to identify AD patients earlier in disease progression, allowing for a timely pharmacological intervention. We believe that Amprion's technology offers a solution to this problem.

The value of our technology platform lies in our ability to detect soluble amyloid-β oligomers directly with high sensitivity and selectivity. Our preliminary results also indicate that soluble amyloid-β oligomers are released from the CNS to the blood, and can be detected in serum or plasma. This offers the opportunity to develop a blood-based, sensitive diagnostic for AD that is suitable for routine clinical screening in a large number of patients. In contrast to blood samples, CSF sample generation is expensive, relatively complicated, and has potential risk of significant complications. Assay technologies relying on CSF-based measure can therefore never achieve the routine use that is possible with a simple blood-based diagnostic.

Our technology platform detects the critical molecular pathogenic event directly, rather than relying on indirect measures such as total pool of amyloid-β peptides, of which only a small fraction forms the synapto-toxic oligomeric aggregates. We therefore believe that our technology offers the best opportunity to develop a diagnostic test that is able to diagnose the disease prior to the onset of significant cognitive symptoms. The purpose of this STTR grant application is to generate data to further support this contention for AD.

Many therapeutic approaches for AD currently under investigation target amyloid-β species, either directly or indirectly. Our platform offers a biomarker approach to complement these therapeutic strategies. Patients with high levels of amyloid-β oligomers, and thus most likely to respond to a treatment targeting this pathogenic even, can be identified using our technology. Our platform can also serve as a primary or secondary outcome measure, following the reduction of amyloid-β species through the course of a treatment.

Furthermore, the formation and accumulation of misfolded proteins is a hallmark event of various diseases other than AD, including highly prevalent illnesses such as Parkinson's disease and type 2 diabetes. Therefore, our findings may enable to create a platform technology to develop tests for detection of other protein misfolding diseases.

III. COMPANY

Amprion Inc is a start-up biotechnology company specialized in the development of novel technologies for the detection of misfolded proteins associated with a number of human and animal diseases, including some of the most prevalent neurodegenerative diseases. The company focuses on using our patented technologies to amplify a very low number of abnormally folded proteins to make them detectable by standard methods such as western blot and ELISA. Amprion's core technology, developed by Prof. Claudio Soto, allow extremely sensitive detection of misfolded proteins at levels several million fold lower than currently used technologies while retaining very high levels of specificity approaching 95%. Amprion's highly novel and scientifically-vetted technology can provide very early detection for slowly evolving neurodegenerative diseases and allow both more efficient screening of blood and infected animals as well as facilitate early treatment and monitoring for these diseases. The potential market for an effective early-detection method that tracks progression of these diseases is staggeringly large.

Amprion Inc is based in Houston and San Diego, and was created in partnership with the University of Texas in 1996. [redacted] our own laboratories in San Diego, where Amprion's management team is based. [redacted]

In 2012, Amprion was awarded a Phase I/Phase II Fast Track STTR grant for [redacted] to develop its technology specifically for prion disorders.

Amprion management team consists of:

Dr Russell Lebovitz, President and CEO. Dr Lebovitz has broad experience in commercialization of early-stage life-science technologies, strong relationships with institutional venture investors, pharmaceutical-industry thought leaders, leading clinicians in North America, Europe, and worldwide. Dr Lebovitz has extensive experience in managing biotech companies, especially in the diagnostic area. During the last decade, Dr. Lebovitz has managed global projects for a number of Fortune 100 technology companies as well as leading venture capital and investment banking firms. He has helped raise over $100 million for early and intermediate stage pharmaceutical and biomedical device companies. Prior to his business activities, Dr. Lebovitz worked for over fifteen years as a successful senior scientist and research physician. Dr. Lebovitz also served on the faculty of Baylor College of Medicine in Houston and The

Fox Chase Cancer Center in Philadelphia. He also has experience in successfully applying and managing NIH SBIR/STTR programs as Principal Investigator

Dr Claudio Soto, Scientific Founder, Vice-President and Chief Scientific Officer. He is currently Professor of Neurology and Director of the George and Cynthia Mitchell Center for Alzheimer's disease and related Brain Disorders at the University of Texas Medical School in Houston. After receiving his PhD from the University of Chile and performing postdocs at the Catholic University of Chile and at the New York University School of Medicine, he became an assistant professor in 1995. In 1997, he participated in the creation of the New York-based biotech company Axonyx Inc, which became later trading in Nasdaq and reached a peak valuation of more than 300 millions dollars. Between 1999 and 2003, Dr Soto was Senior Scientist, Chairman of the Department of Molecular Neurobiology and Senior Executive Scientific Advisor for Neurology at Serono International in Switzerland. [redacted] Between 2003 and 2008, he served as Director of the George and Cynthia Mitchell Center for Neurodegenerative Diseases and Professor on the Departments of Neurology, Neuroscience & Cell Biology and Biochemistry & Molecular Biology at the University of Texas Medical Branch in Galveston. For the past 19 years, he and his colleagues have engaged in research into the molecular basis of neurodegenerative diseases associated to the misfolding and brain accumulation of proteins, particularly focusing in Alzheimer's and prion-related disorders. His work has led to the development of novel strategies for treatment and diagnosis of these diseases. He has published more than 140 peer review scientific publications and contributed to more than 20 books, including one written entirely by Dr. Soto. Many of his studies have been published in the most prestigious scientific journals (including Cell, Nature, Science, Nature medicine, The Lancet, PNAS, Neuron, EMBO J, etc). Dr Soto has received numerous awards and has been invited speaker in more than 150 International scientific meetings worldwide. He has been awarded many grants from NIH, and private foundations [redacted]. Dr Soto's discoveries have been extensively highlighted in the scientific literature and lay media with several hundreds of articles published in newspapers, magazines, TV and radio worldwide about his work.

Dr. Benedikt Vollrath, Chief Operating Officer. Dr. Vollrath is an experience life science executive with over 14 years of experience in pharmaceutical and biotechnology companies. Prior to his engagement with Amprion, he spent over 4 years as Entrepreneur in Residence at Avalon Ventures, a San Diego-based Venture Capital firm focused on developing early-stage life science companies. During his tenure with Avalon Ventures, Dr. Vollrath was a co-founder and senior member of the management

teams of several biotech companies, incluing Afraxis (focused on CNS therapeutics, subsequently sold to Genentech for $187M), Carolus Therapeutics (focused on therapeutics for inflammatory diseases) and Otonomy (focused on therapeutics for diseases of the inner and middle ear). Prior to Avalon, Dr. Vollrath was a Scientist at Renovis, a San Francisco-based CNS drug discovery company, and Merck. Dr. Vollrath has broad experience in managing virtual and semi-virtual research programs, and is inventor on over 40 patents or patent applications.

III. MARKET, CUSTOMER, AND COMPETITION

Current estimates suggest that 5.2 million Americans have Alzheimer's Disease in 2012, and this prevalence is expected to increase dramatically as populations age. By 2050, as many as 15 million Americans are expected to have AD, unless breakthrough medicines can halt or reverse the disease.

We see two main applications of our diagnostic platform: first, our technology platform can serve as a companion diagnostic for drug development and clinical practice. The majority of therapeutic approaches for AD pursued at the moment target amyloid-β oligomer homeostasis, either directly or indirectly. We believe that our platform can identify patients suitable for amyloid-β-targeting therapies, and can discriminate responders from non-responders. Two recent large-scale Phase III trials investigating novel modulators of amyloid-β homeostasis ended in failure. Inclusion criteria for these clinical studies were based exclusively on cognitive scales, and measures of aberrant amyloid-β homeostasis were not included. Categorizing patients into AD- and non-AD dementia using cognitive scores alone has an inherent error rate, and treating patients with amyloid-β modifying therapies is not expected to show efficacy if there is no underlying defect in amyloid-β homeostasis. We believe that our platform can identify patients that show aberrant amyloid-β homeostasis, and are thus most likely to respond to treatments targeting this defect, using a blood-based assay. This represents a critical missing piece in clinical trial design, and we believe that adding this measure as an inclusion criterium will greatly increase the chances of success in clinical trials for novel AD therapeutics.

Second, because our technology detects the postulated pathogenic species - soluble amyloid-β oligomers - with extremely high sensitivity, we believe that this technology offers the best opportunity to identify AD patients at the earliest stages, possibly before clinical symptoms of cognitive decline set in. In this application, our diagnostic platform would be used as a screening tool in older patients, perhaps starting at the age of 55 or 60 years of age. Identifying potential AD patients early, and possibly before onset of significant clinical symptoms and cognitive decline, greatly enhances the chance of effective therapeutic intervention, independent of the mechanism of action of

the therapeutic. The screening market to identify AD patients early will be very high once appropriate therapeutics are on the market that can be a suitable response to a positive test.

The first and primary customers for the companion diagnostic market are most likely to be large pharmaceutical or biotechnology companies developing novel therapeutics for AD that wish to include novel biomarker technology to their clinical development program as companion diagnostics. This market is best accessed by Amprion through licensing agreements in which the therapeutics developer gains access to the technology in exchange for fees and a royalty stream. While drug development companies do not need a license from Amprion during clinical trials for internal studies because of the safe harbor exemption, in our experience potential pharma and biotech partners will be careful to secure the rights to the technology before incorporating any outcome measures in their clinical development plan. We therefore believe that potential partners will engage early with Amprion, and this STTR grant application is designed to produce the data necessary to secure these transactions. Even at the current state of development, Amprion was able to secure an exploratory collaboration with a biotech company developing novel therapeutics for AD by targeting soluble amyloid-β oligomers.

As therapeutic options become available to patients, we believe that screening technologies that identify AD patients early, or identify patients at high risk of developing AD in the near future, or identify responders and non-responders to a particular therapeutic regimen will become increasingly important. We believe that our technology can provide a solution to this problem. For this second market, customers will be the patients themselves, or the insurance companies and government programs insuring those patients.

There is currently no biochemical assay either in CSF or blood samples that is used in routine clinical practice to diagnose AD or track disease progression. Several technology platforms are attempting to address this problem, but so far all offerings require CSF samples, show limited selectivity and specificity, and cannot reliably identify AD patients prior to onset of cognitive symptoms.

Amyloid-β assays. Several commercial suppliers [redacted] are offering ELISA-based assays to detect the total levels of amyloid-β peptides (most commonly amyloid-β 1-42) as a biomarker for AD. In some settings, this assay is combined with ELISA-based measures of phospho-tau, another potential disease marker for AD. Currently existing amyloid-β platforms have several significant limitations that restrict their use in clinical practice: (1) these diagnostic platforms detect the *total* pool of amyloid-β peptides, which includes both monomeric peptides as well as soluble oligomers. Monomeric amyloid-β is not thought to be damaging to synapses and neurons, and only the oligomeric aggregates are regarded as the synapto-toxic amyloid-

β species. A very small portion of the total amyloid-β pool forms aggregates. While this measure has shown some correlation with cognitive scores, the selectivity and specificity in AD diagnosis is not very high. Furthermore, the level of amyloid-β 1-42 in CSF of patients is surprisingly lower than in controls, and reason for this observation is currently unknown.By using methodologies such as Amprion's that are specific for oligomeric amyloid-β, these limitations can potentially be overcome. (2) All currently available ELISA-based diagnostics require CSF, which contains higher levels of amyloid-β peptides and aggregates. Generating CSF samples is relatively invasive, subject to potential severe complications and thus not suitable for routine screening and diagnosis. (3) Currently, none of the ELISA-based methods described offer sufficient sensitivity and specificity to detect changes in amyloid-β homeostasis in early AD patients that do not yet show cognitive decline. We believe that our technology platform, which specifically amplifies oligomeric amyloid-β aggregates, offers a solution to all these three limitations.

[redacted] This technology assesses 16 different protein species, including amyloid-β, using a platform based on mass spectroscopy. While mass spectroscopy is capable for detecting very small amounts of target analyte, the method is limited in its throughput, and not suitable for high-volume screening of samples. In addition, this platform, like the other platform technologies outlined above, measures total amyloid-β peptide levels, not the levels of the pathogenic species. In our evaluation, it therefore suffers from the same limitations regarding specificity, sensitivity and ability to detect changes in the relevant amyloid-β oligomers as the ELISA-based methods currently used. The relationship of the other 15 protein species analyzed by this platform to the pathogenesis of AD is unclear at this point, and the correlation observed between AD and measures in these proteins might very well represent epi-phenomena with no causal relationship to AD pathogenesis.

INTELLECTUAL PROPERTY PROTECTION

Amprion holds the rights or has an exclusive license to a wide array of patents and patent applications covering the PMCA technology. There are two granted patents covering the PMCA technology and one additional filed application currently under review.

We have a very close and legally documented relationship with the University of Texas Health Science Center in Houston for all Dr Soto's know-how and technologies in the area of detection of protein misfolding disorders. We have a group of patent attorneys that frequently work with us on pursuing our files and protecting our intellectual property. We mostly work with [redacted] at the prestigious law firm [redacted]

For any new IP generated during the course of this project, we will follow standard NIH guidelines and US laws covering patent protection and inventorship. Where appropriate, patent filing will be closely coordinated between Amprion and the University of Texas Health Science Center. The patent prosecution costs will be covered by Amprion in return for a license covering the commercialization of the novel technology.

V. FINANCE PLAN

The costs associated with validating the platform for AD diagnosis, and compiling data to support the utility of this technology both for identifying early-stage, pre-symptomatic AD patients as well as a biomarker for clinical studies, can be borne to by this STTR grant. However, additional resources will be required to generate the data necessary for full regulatory approval of this test by the FDA and other regulatory bodies in the EU and abroad.

We believe that one or multiple collaboration agreements with large biotechnology or pharmaceutical companies that have ongoing programs for novel therapeutics for the treatment of AD are a fruitful avenue to raise the funds necessary for further development past the activities outlined in this proposal. Co-development transactions, where a large company partner provides financial and in-kind resources to develop further develop the platform in exchange for licensing rights to our patent-protected platform are a tested and attractive avenue to raise capital for a company at our stage of development. Even at this early stage of development of the asset, we have already entered into one exploratory collaboration agreement with a company developing novel therapeutics targeting amyloid-β. This existing relationship is non-exclusive, and we strongly believe that we can form other such agreements as we develop this platform. Data generated under this STTR grant will put us in a position to

aggressively pursue such partnership deals. In our evaluation, this avenue should be the primary focus to raise additional resources to fund the development of this program, and we are actively pursing this approach already.

An alternate route is to seek traditional Venture Capital (VC) funding to advance the asset to regulatory approval and beyond. Both Dr. Lebovitz and Dr. Vollrath are well connected in the VC community and have the personal relationships necessary to pursue a fund-raising campaign with risk-capital funds. However, funding for early-stage innovation is very difficult to obtain at the moment: many health-care focused VC funds are seeking later stage deals or have raised smaller funds post-recession. While this might change during the course of this STTR grant proposal, especially with one or a few large break-through “wins” that increases the appetite for risk with investors, we nevertheless regard this second avenue as challenging, at least for the moment. The current atmosphere in VC funds, with a few notable exceptions, is largely risk-adverse, even for a technology platform such as Amprion’s that is comparatively low-risk and low-cost compared to the much higher stakes of a drug development effort. However, we will continue to observe the VC investment climate, and continue to discuss our programs with partners and colleagues that manage VC funds.

The funds necessary for development through regulatory approval after this STTR grant project are in an order of magnitude suitable for “angel investors”. As Director of the Mitchell Center for Alzheimer’s disease and related Brain disorders, Dr Soto has a network of connections with potential philanthropists that could be approached for such a project. [redacted] In addition, Drs. Lebovitz and Vollrath are well connected with angel investor networks [redacted] and will continuously probe this network of relationships for investors suitable to support our programs.

VI. PRODUCTION AND MARKETING PLAN

For the first phase of developing the companion diagnostic market, we plan to set up our own CLIA-compliant lab to test clinical samples from pharma and academic partners. The PMCA assay, although simple and conceptually very straightforward, requires experience and specific know-how to be done in a highly reproducible way. The high power of amplification which is essential for ultra-sensitive detection of amyloid-β oligomers, can also amplify artifacts introduced by technicians in the lab. These issues have been successfully addressed by other amplification based diagnostic tests such as PCR. Furthermore, there is always a potential for cross-contamination that

has to be minimized to the minimum by employing good laboratory practices. We believe that setting up a single, CLIA-compliant laboratory is the most straightforward way to allow testing these relatively small number of clinical samples during clinical trials. In the second phase of developing this market, as the companion diagnostic market becomes more established, and more drug development programs use Amprion's technology platform to identify suitable patients and to follow disease progression, we will consider out-licensing the technology to large full-service lab service providers that already have an infrastructure in place to handle large numbers of blood samples.

Should a pharma partner wish to enter into an exclusive licensing transaction with Amprion, we might also consider aiding the set-up of an Amyloid-β PMCA platform in the laboratories of our partners. Amprion personnel would be involved in the transfer of the technology to our partners.

If the Amprion platform becomes a routine screening platform to identify pre-symptomatic AD patients prior to onset of cognitive symptoms, we will consider out-licensing the technology to lab service providers that already have the capabilities in place to handle, track and data manage large numbers of blood samples. The number of samples that need to be assessed in this scenario will almost certainly overwhelm the capacities that we can reasonably set up in-house, and we will rely on the expertise of established participants in the blood screening market to offer our technology to patients.

VII. REVENUE STREAM

During the first phase of developing the companion diagnostic market, we are expecting to be able to command non-exclusive licensing fees [redacted] per transaction for access to the technology. We also anticipate charging an additional fee of [redacted] per clinical sample for performing and validating the tests. During the clinical development of a drug candidate, this would yield revenues between [redacted] per transaction. We estimate that the costs for running the tests (including personnel, laboratory facilities, reagents and shipping costs) to be between [redacted] of the total revenue, and we anticipate to invest the profit into R&D to develop our platform for other misfolded protein disorders.

We believe that in the following stage of market development, revenue for Amprion will mainly come from licensing agreements and royalty stream. Potential licensing partners are expected to be large pharma or biotechnology companies with therapeutics for AD on the market, or lab services providers that offer various clinical tests to hospitals and doctors. It is also possible that either Amprion's amyloid-β PMCA platform, or the entire company will be sold. [redacted]

[redacted] While the promise of this PET ligand has not been realized as plaque load itself is now regarded as a poor marker for disease progression in AD, and certainly a very late event in the disease and thus unable to identify early, pre-symtomatic patients, this transaction can give insight into the value the industry can place on biomarkers for AD.

OMB Number: 0925-0001

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page. The response provided on that page, regarding the type of application being submitted, is repeated for your reference, as you attach the appropriate sections of the Research Plan.

*Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)		Add Attachment	Delete Attachment	View Attachment
2. Specific Aims	1242-Specific aims.pdf	Add Attachment	Delete Attachment	View Attachment
3. *Research Strategy	1243-Research plan.pdf	Add Attachment	Delete Attachment	View Attachment
4. Inclusion Enrollment Report		Add Attachment	Delete Attachment	View Attachment
5. Progress Report Publication List		Add Attachment	Delete Attachment	View Attachment

Human Subjects Sections

6. Protection of Human Subjects	1244-Human subjects.pdf	Add Attachment	Delete Attachment	View Attachment
7. Inclusion of Women and Minorities		Add Attachment	Delete Attachment	View Attachment
8. Targeted/Planned Enrollment Table		Add Attachment	Delete Attachment	View Attachment
9. Inclusion of Children		Add Attachment	Delete Attachment	View Attachment

Other Research Plan Sections

10. Vertebrate Animals	1245-Vertebrate animals.pdf	Add Attachment	Delete Attachment	View Attachment
11. Select Agent Research		Add Attachment	Delete Attachment	View Attachment
12. Multiple PD/PI Leadership Plan	1246-MultiplePI_LeadershipP	Add Attachment	Delete Attachment	View Attachment
13. Consortium/Contractual Arrangements	1247-LOI UT.pdf	Add Attachment	Delete Attachment	View Attachment
14. Letters of Support	1248-letters of support.pdf	Add Attachment	Delete Attachment	View Attachment
15. Resource Sharing Plan(s)		Add Attachment	Delete Attachment	View Attachment

16. Appendix Add Attachments | Remove Attachments | View Attachments

SPECIFIC AIMS

This is a Fast-track application for the STTR program. Alzheimer's disease (AD) is the most common dementia in the elderly population and one of the leading causes of death in the developed world. One of the main problems in AD is the lack of an early, sensitive and objective laboratory diagnosis to identify individuals that will develop the disease before substantial brain damage. Compelling evidence indicates that the hallmark event in AD is the misfolding, aggregation and brain accumulation of amyloid-beta (Aβ) protein. Aβ aggregation follows a seeding-nucleation mechanism and involves several intermediates, including soluble oligomers and protofibrils [1;2]. Recent evidence has shown that Aβ oligomers are circulating in biological fluids and these structures appear to be key for inducing brain degeneration in AD [3;4]. Thus, it seems that detection of soluble Aβ oligomers might represent the best strategy for early and specific biochemical diagnosis of AD. The challenge with this approach is that the quantity of Aβ oligomers is likely very small in tissues other than the brain and their sequence and chemical structure is the same as the more abundant native Aβ protein.

Our working hypothesis is that detection of misfolded Aβ oligomers circulating in blood may be the basis for an early biochemical diagnosis for AD. Our approach is to use the functional property of misfolded oligomers to catalyze the polymerization of the monomeric protein as a way to amplify them, thus greatly facilitating their detection and characterization. We have recently invented the protein misfolding cyclic amplification (PMCA), which represents a platform technology to detect very small quantities of seeding-competent misfolded oligomeric proteins associated with various protein misfolding diseases [5-8]. Currently, PMCA has been optimized to detect low levels of misfolded prion protein implicated in prion diseases in various biological fluids, including blood and urine [7;9-11] and has more recently been shown to amplify soluble Aβ oligomers in cerebrospinal fluid of AD patients [8]. The major goal of this project is to adapt the PMCA technology for the specific and sensitive detection of misfolded Aβ oligomers in human blood, perform studies of specificity and sensitivity using large number of samples and evaluate the utility of Aβ-PMCA for pre-clinical identification of people on the way to developing AD. The results generated in this project may lead to the first biochemical test for blood-based diagnosis of AD with both high sensitivity and specificity. The studies included in this project will constitute the basis for regulatory approval of the test that Amprion will commercialize.

Phase I objectives and milestones

Specific Aim 1. Optimization of the experimental conditions for detection of Aβ oligomers in AD blood. The studies in this aim are designed to optimize the Aβ-PMCA assay conditions for detection of Aβ oligomers in human blood. We will use the knowledge and experience gained in optimizing the Aβ-PMCA conditions for detection of Aβ oligomers in CSF as well as our successful use of the prion PMCA technology to detect misfolded prions in blood. The plan is to first optimize the technique using healthy plasma samples spiked with *in vitro* generated Aβ oligomers. When reproducible detection of low quantities of synthetic oligomers is achieved, we will switch to work with plasma samples from well-characterized AD patients and transgenic mice models of AD. The main goal of phase I is to optimize the technology for high reproducibility as well as to estimate our detection sensitivity limit, specificity and the cut-off for the assay. We will also do a pilot test using a small number of human AD samples. The milestone set for this phase is to have an optimized technology for reproducible detection of at least 1 $x10^{-15}$ moles of Aβ oligomers in plasma and be able to detect Aβ oligomers in AD blood plasma in a small number of patient samples.

Phase II objectives and milestones

Specific Aim 2. Evaluation of the sensitivity and specificity in large number of plasma samples from AD patients. To investigate the usefulness of the Aβ-PMCA technology for AD diagnosis, we will test blood plasma samples from large number of patients with AD diagnosis (at least 500), as well as age-matched healthy controls, young healthy individuals and people affected by other forms of dementia and neurological diseases. Samples will be provided blinded from at least 3 different Institutions. Sensitivity, specificity, and positive and negative predictive values will be estimated by receiving operating curve (ROC) analysis. The milestone for this aim is to achieve at least 90% sensitivity and specificity in the large panel of samples.

Specific Aim 3. Study the usefulness of Aβ-PMCA to monitor disease progression and for pre-clinical diagnosis of AD. To assess whether Aβ oligomers can be specifically detected in people at high risk for developing AD, we will use samples from persons affected by mild cognitive impairment as well as asymptomatic carriers of familial AD mutations. To study in more detail the earliest time in which Aβ oligomers are detectable in plasma before the massive deposition of cerebral Aβ plaques, we will do a time-course experiment in a transgenic animal model of AD. To evaluate whether detection of Aβ oligomers in biological fluids (both CSF and plasma) correlates with the stage of AD and can be used to monitor disease progression, we will test samples from patients at various clinical stages of AD and will also do experiments with longitudinal samples from the same patient collected at different times. Finally, we will study the correlation between the Aβ oligomers in CSF and blood from the same patients.

The main milestone for the studies included in phase II will be to satisfy the requirements of the regulatory authorities to obtain approval for a blood test for detecting and predicting the clinical course of AD.

RESEARCH STRATEGY

SIGNIFICANCE

AD is a devastating degenerative disorder of the brain for which there is no effective treatment or accurate pre-clinical diagnosis [12]. The disease is characterized by memory loss, confusion and a variety of cognitive disabilities. The major neuropathological changes in the brain of AD patients are neuronal death, synaptic alterations, brain inflammation and the presence of protein aggregates in the form of extracellular amyloid plaques and intracellular neurofibrillary tangles [13]. Although the etiology of the disease is not yet clear, compelling evidence suggest that the misfolding, aggregation and brain deposition of the amyloid-beta protein (Aβ) is the triggering factor of the pathology [14-16].

The study of the structural and molecular mechanisms of Aβ fibrillogenesis have been extensively pursued by many groups [1;16-18]. Aβ misfolding and fibrillar aggregation follow a seeding-nucleation mechanism that involves the formation of several intermediates, including soluble oligomers and protofibrils [1;2;19]. Recent findings have shown that Aβ oligomers, rather than large amyloid fibrils, might be the culprit of neurodegeneration in AD [3;15;20;21]. AD belongs to a large group of diseases associated with misfolding, aggregation and tissue accumulation of proteins [22]. These diseases, termed protein misfolding disorders (PMDs), include Parkinson's disease, type 2 diabetes, Huntington's disease, amyotrophic lateral sclerosis, systemic amyloidosis, prion diseases, and many others [22;23]. In all these diseases, misfolded aggregates composed of different proteins are formed by a similar mechanism resulting in the accumulation of toxic structures that induce cellular dysfunction and tissue damage [1;2;19].

One of the major problems in AD is the lack of a widely accepted early, sensitive and objective laboratory diagnosis to support neuropsychological evaluation, monitor disease progression and identify affected individuals before they display clinical symptoms [24;25]. For diseases affecting the brain, a tissue with low regeneration capacity, it is crucial to intervene before irreversible neuropathological changes occur. Therefore, early diagnosis of AD is of utmost importance. Currently, diagnosis of probable or possible AD is done mostly by clinical examination complemented by some imaging techniques that are used mainly to rule out other forms of dementia [26]. In recent years, the measurement of the levels of total Aβ42, total Tau and phosphorylated Tau in CSF have been shown to give a good sensitivity and specificity and help in the diagnosis of AD [27-29]. Definitive diagnosis is done post-mortem by histological examination of the brain for the presence of amyloid plaques and neurofibrillary tangles. Despite much effort to identify biochemical markers for the disease, there are still not accepted surrogate markers other than the misfolded aggregated proteins. Several lines of evidence indicate that the process of Aβ misfolding and oligomerization begins years or decades before the onset of clinical symptoms and substantial brain damage [30;31]. Recent studies have shown that Aβ oligomers are naturally secreted by cells and circulate in AD biological fluids [32-37]. Thus, detection of soluble Aβ oligomers might represent the best strategy for early and specific biochemical diagnosis of AD.

Our strategy to detect misfolded oligomers is to use their inherent ability to catalyze the polymerization of the monomeric protein. For this purpose, we recently invented the protein misfolding cyclic amplification (PMCA), which is a platform technology that enables ultra-sensitive detection of misfolded aggregates through acceleration and amplification of the misfolding and aggregation process *in vitro* [5;6]. The basis for the PMCA technology is the fact that the processes of misfolding and aggregation of Aβ, and all the other proteins implicated in PMDs, follow a seeding-nucleation mechanism [38-40]. PMCA is a cyclical process; each cycle is composed of two phases. During the first phase the samples containing minute amounts of misfolded oligomers and an excess of monomeric protein are incubated to induce growth of the polymers by converting soluble monomeric protein to polymeric misfolded protein. In the second phase the sample is subjected to sonication to break down the polymers, multiplying the number of seeding-competent nuclei [6]. After each cycle the number of seeds increases in an exponential fashion. PMCA is conceptually analogous to DNA amplification by PCR. In both systems a template grows at the expense of a substrate in a cyclic reaction, combining growing and multiplication of the template units.

The PMCA technique has already been successfully applied to amplify and detect misfolded prion protein (PrP^{Sc}) implicated in prion diseases [5;41]. Using PMCA, we were able to detect the equivalent of a single particle of misfolded PrP oligomers [41] and strikingly, to identify PrP^{Sc} in the blood and urine of infected animals at symptomatic and pre-symptomatic stages of the disease [9-11]. In a very recent article, we reported the expansion and adaptation of PMCA to amplify seeding-competent Aβ oligomers circulating in the CSF of AD patients and its application to distinguish with high sensitivity and specificity samples coming from AD patients from other forms of dementia or neurodegenerative diseases [8].

The major goal of this project is to develop and validate an early, objective and sensitive blood test for AD diagnosis. In recent years much progress has been done to understand the molecular basis of AD and the

development of novel strategies for treatment [42;43]. However, we still lack effective ways to prevent, cure or even slow the progression of AD. Part of the difficulties in developing an efficient treatment for this devastating disease is the absence of an early and objective biochemical diagnosis that will enable us to recognize patients before substantial brain damage has occurred [24;25;44]. Indeed, many of the failures of recent clinical trials with compounds aimed at modifying the disease progression have been attributed to the lack of an early diagnosis that would permit treatment to begin before damage is irreversible [45]. Considering the low capacity of the brain to regenerate itself, it is very likely that any therapy will have the most benefit if treatment is started prior to significant brain damage. An accurate and reliable biochemical diagnostic procedure will also be useful for monitoring the progression of the disease, establishing the efficacy of novel treatments and understanding their potential mechanisms of action.

INNOVATION

The proposed research project is expected to lead to the development of a blood test for AD that may be useful not only to aid in the diagnosis of the disease, but also to identify people on the way to developing AD pathology before the onset of substantial brain damage and clinical symptoms of the disease. There are several innovative aspects of this project:

- The technological development and adaptation for the AD field of the revolutionary PMCA technique that has the potential to amplify and detect human misfolded proteins down to the level of even a single copy of the marker [41]. Application of this “protein-based PCR-like” technology for the development of a blood test for AD is a highly innovative aspect of this project.
- Our technology platform detects the critical molecular pathogenic event directly, rather than relying on indirect measures such as non-pathogenic biomarkers or the total pool of amyloid-β peptides, of which only a small fraction forms the synapto-toxic oligomeric aggregates.
- We have a highly dynamic and flexible strategy for commercialization as described in the “Commercialization Plan” which includes exploring simultaneously different applications, markets and customers. We see two main applications of our blood test. First, our technology platform can serve as a companion diagnostic for drug development and clinical practice. The primary customer for the companion diagnostic market will be large pharmaceutical or biotechnology companies developing novel therapeutics for AD that wish to take advantage of our technology as an integral part of their clinical development program. Second, our technology offers the best opportunity to identify AD patients at the earliest stages, possibly before clinical symptoms of cognitive decline set in. In this application, our diagnostic platform would be used as a screening tool in older or at-risk patients, perhaps starting at the age of 55 or 60 years of age.
- We have a very knowledgeable and active management team that has all the relevant expertise to accomplish the scientific and technical development, regulatory approval and commercialization of the technology. Dr. Russ Lebovitz (CEO) has been managing several biotech companies in the diagnostic area and has directly risen over $100 million for early and intermediate stage pharmaceutical and biomedical device companies. He has broad experience in commercialization of early-stage life-science technologies, strong relationships with institutional venture investors, pharmaceutical-industry thought leaders, and leading clinicians in North America, Europe, and worldwide. Dr. Benedikt Vollrath (COO) is an experienced life science executive with over 14 years of experience in pharmaceutical and biotechnology companies. Dr. Vollrath was a co-founder and senior member of the management teams of several biotech companies, incluing Afraxis (focused on CNS therapeutics, subsequently sold to Genentech for $187M), Carolus Therapeutics (focused on treatments for inflammatory diseases) and Otonomy (focused on therapeutics for diseases of the inner and middle ear). Dr Claudio Soto (CSO) is a widely recognized scientific leader in the field of protein misfolding diseases, who has produced many important contributions to the field. Dr Soto also has substantial experience in the translation and commercialization of scientific discoveries. In 1997, he participated in the creation of the New York-based biotech company Axonyx Inc, which later traded on Nasdaq and reached a peak valuation of more than 300 millions dollars. He was also formerly in charge of the neurology portfolio of the largest European biotech company (Serono), who successfully took a compound discovered by Dr Soto to human clinical trials in the field of AD.
- Formation and accumulation of misfolded proteins is a hallmark event of various diseases, including highly prevalent disorders such as Alzheimer’s, Parkinson’s disease and type 2 diabetes [46;47]. Therefore, our findings may enable the creation of a platform technology to develop tests for detection of other protein misfolding diseases [6]. Indeed, our recent preliminary studies have shown that in addition to prion protein and Aβ protein, PMCA can be adapted for high sensitive and specific detection of

oligomeric, misfolded alpha-synuclein protein present in the CSF of patients affected by Parkinson's disease (Soto et al., unpublished results). Development of a PMCA-based test for biochemical diagnosis of Parkinson's disease as well as some of the other highly prevalent protein misfolding disorders is a very interesting possibility that will be pursued separately. Nevertheless, the findings and technological improvements obtained in this project will certainly be very beneficial for the development of a test for other protein misfolding diseases.

PRELIMINARY RESULTS

Sensitive detection of misfolded Aβ oligomers. To implement the experimental conditions for cyclic amplification of Aβ misfolding (Aβ-PMCA), we performed studies using *in vitro* produced oligomeric seeds. Since it is unknown which of the different species of Aβ oligomers is most relevant for AD pathology, we decided to work with a mixture of oligomers of different sizes generated during the process of fibril formation. Aβ oligomers were prepared by incubation of monomeric (seed-free) synthetic Aβ42 (10 μM) at 25 ºC with stirring. After 5 h of incubation, we observed an abundance of globular oligomers by electron microscopy with only a small amount of protofibrils and fibrils (Fig. 1A). These aggregates were positive with the A11 anti-oligomer specific antibody [48] (data not shown). The size of the aggregates was determined using filters of defined pore size and western blotting after SDS-PAGE separation. Oligomers formed by incubation for 5 h were retained in filters of 30 kDa cut-off and passed through in 1000 kDa cutoff filters (not shown) and migrated as ~170 kDa SDS-resistant aggregates, with a minor band at 17 kDa (Fig. 1B). Low concentrations of seed-free Aβ42 (2 μM) were incubated at 22 ºC with constant stirring (100 rpm) for different times alone or in the presence of distinct concentrations of synthetic Aβ oligomers (Fig. 1C). Aβ aggregation was studied by the fluorescence emission of the amyloid-binding dye thioflavin T (ThT) [49;50]. Under these conditions, no spontaneous Aβ aggregation was detectable during the time in which the experiment was performed (125 h). However, Aβ aggregation was observed under these conditions in the presence of 0.3 to 8.4 pmol of Aβ oligomers (Fig. 1C). To increase the efficiency of seeding, and thus the limit of detection of Aβ oligomers, we introduced cycles of amplification, combining phases of polymer growth with multiplication of seeds as in the PMCA assay. For this purpose, we subjected the samples to intermittent shaking, which has been shown to dramatically accelerate the seeded conversion of recombinant prion protein [51]. Under these conditions, the kinetics of Aβ aggregation induced by 8400, 300, 80 and 3 fmol of Aβ oligomers was clearly faster and easily distinguishable from that observed in the absence of Aβ seeds (Fig. 1D). This result indicates that using the Aβ-PMCA assay we should be able to detect as little as 3 fmol of Aβ oligomers.

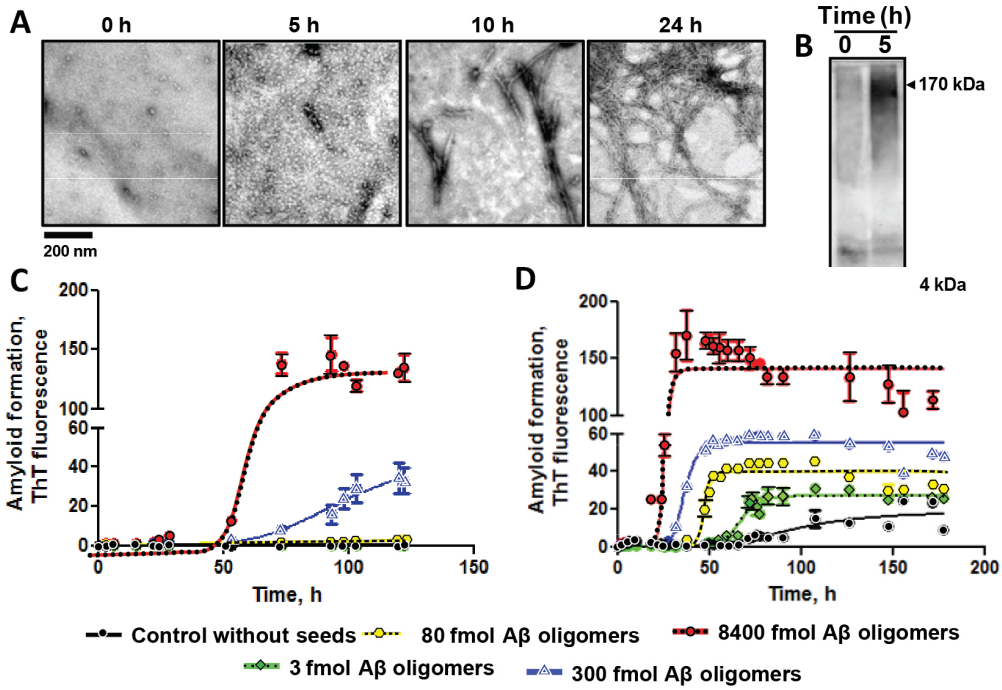

Fig. 1. Detection of synthetic Aβ oligomers by Aβ-PMCA. **A:** Aβ oligomers were prepared as described

Fig. 1. Detection of synthetic Aβ oligomers by Aβ-PMCA. **A:** Aβ oligomers were prepared as described in the text and their identity was checked by electron microcopy after negative staining after different times of incubation. **B:** Preparations of oligomers were characterized by SDS-PAGE followed by western blot with 4G8 antibody. **C:** Seeding of Aβ aggregation was studied incubating a solution of 2 μM seed-free Aβ1-42 in 100 mM Tris-HCl pH 7.4, in the presence of 5 μM Thioflavin T, with or without different quantities of synthetic Aβ oligomers with constant, but low agitation (100 rpm) at 22 ºC. **D:** The same samples as in panel C were incubated with cyclic agitation (1 min stirring at 500 rpm followed by 29 min without shaking). Aggregation was measured over time by the thioflavin T (ThT) assay Graphs show the mean and standard error of 3 replicates. The concentration of Aβ oligomers was estimated assuming an average molecular weight of 170 kDa.

Detection of Aβ Oligomers in the CSF of AD Patients. To study the usefulness of the Aβ-PMCA assay to detect seeding-competent Aβ oligomers in biological fluids, we analyzed aliquots of CSF from 50 AD patients, 39 cognitively normal individuals affected by non-degenerative neurological diseases (NND), and 37 patients affected by non-AD neurodegenerative diseases including other forms of dementia (NAND). Figure 2A shows the average kinetics of aggregation of 5 representative samples from the AD, NND, and NAND groups. The result indicates that CSF from AD patients accelerates significantly Aβ aggregation as compared with control CSF (P<0.001). To determine the effect of individual samples on Aβ aggregation we estimated the lag phase (Fig. 2B), defined as the time required to get a ThT fluorescence larger than 40 arbitrary units (after subtraction of the blank). This value was selected considering that it corresponds to ~5 times the reading of the buffer alone. We also estimated the P90, which corresponds to the extent of Aβ aggregation at 90 h (Fig. 2C). By comparing both parameters among the experimental groups, a highly significant difference was observed between AD and control samples from individuals with non-degenerative neurological diseases or with non-AD neurodegenerative diseases. No correlation was detected between the aggregation parameters and the age of the AD patients, which indicates that the levels of the marker are not simply a reflection of age, but rather whether or not the patients contain seeding-competent Aβ aggregates in their CSF. Using the values for lag phase, we calculated the sensitivity, specificity and predictive value of the Aβ-PMCA test (Table 1). To determine the appropriate cut-off points and the performance of the test we carried out a detailed statistical analysis of the receiver operating characteristics (ROC). In relation to the control group consisting of age-matched individuals with non-degenerative neurological diseases, we estimated a 90.0% sensitivity and 84.2% specificity, whereas for the clinically more relevant differentiation of AD from other neurodegenerative diseases including other forms of dementia, we obtained a staggering 100% sensitivity and 94.6% specificity (Table 1). If confirmed with a larger

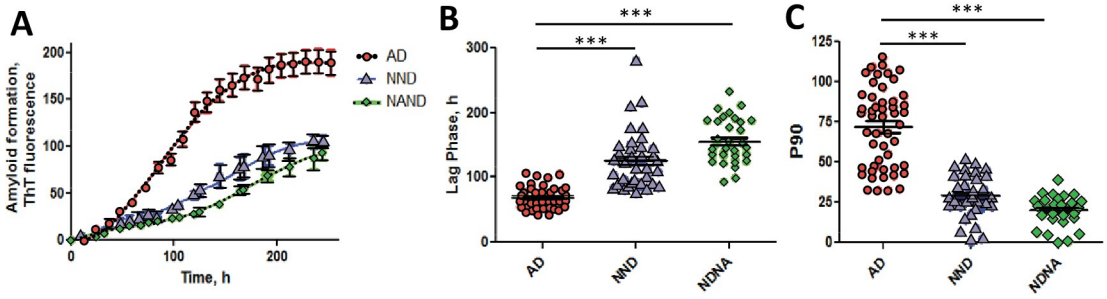

Fig. 2. Detection of seeding activity in human CSF samples from controls and AD patients by Aβ-PMCA. **A:** Representative aggregation curves of seed-free Aβ1-42 in the presence of CSF samples from AD patients, people affected by non-neurodegenerative neurological diseases (NND), and patients suffering from neurodegenerative diseases other than AD (NAND). The values represent the average and standard error of 5 different patients, representative of the average results in each group. **B:** The lag phase of Aβ aggregation in Aβ-PMCA was compared for the 3 groups of patients. Lag phase was defined as the time (in hours) required to reach a ThT signal more than 40. **C:** The extent of amyloid formation obtained after 180 Aβ-PMCA cycles, i.e. 90 h of incubation (P90), was measured in each patient. Data was analyzed by one-way ANOVA, followed by the Tukey's multiple comparison post-test. The differences between AD and the other two groups were highly significant with P<0.001 (***).

Table 1. Estimation of sensitivity, specificity and predictive value for Aβ-PMCA using CSF samples[1]

Groups	Sensitivity[2]	Specificity[2]	Positive Predictive Value[2]	Negative Predictive Value[2]
AD vs NAND	100.0%	94.6%	96.2%	100.0%
AD vs NND	90.0%	84.2%	88.2%	86.5%
AD vs All[3]	90.0%	92.0%	88.2%	93.2%

[1] For estimation of sensitivity, specificity and predictive value we used the results of the lag phase. Cutoffs were estimated by Receiver Operating Characteristics (ROC) curve analysis using the MedCalc software.
[2] Sensitivity was estimated by the formula: [True positives / (True positives + False negatives)] x 100
Specificity was estimated by the formula: [True negatives / (False positives + True negatives)] x 100
Positive predictive value was estimated by the formula: [True positives / (True positives + False positives)] x 100
Negative predictive value was estimated by the formula: [True negatives / (True negatives + False negatives)] x 100
[3] All refers to the samples from NND plus NAND.

number of patients, this ability of Aβ-PMCA to distinguish AD from other forms of neurodegenerative diseases might be very useful in the clinic.

To confirm that Aβ-PMCA detects a seeding activity associated with Aβ oligomers present in CSF, we performed immuno-depletion experiments. The methodology for efficient immuno-depletion of Aβ oligomers was first optimized by using synthetically prepared Aβ oligomers. Incubation with dynabeads conjugated with a mixture of sequence (4G8) and conformational (A11) antibodies led to the complete removal of these structures (Fig. 3A). Next, application of immuno-depletion to 3 AD CSF samples showed that the kinetics of Aβ aggregation in the depleted Aβ-PMCA reaction was comparable to that observed in control CSF samples, and both were significantly different from the aggregation observed with AD CSF prior to immuno-depletion (Fig. 3B). These results indicate that the seeding activity observed in AD CSF samples was indeed associated with Aβ oligomers.

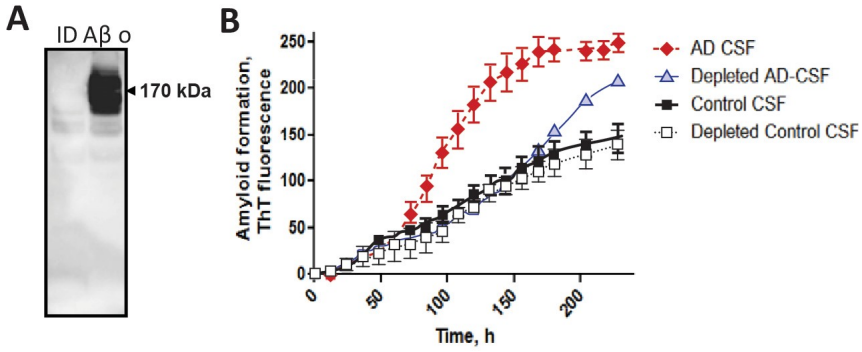

Fig. 3. Seeding activity in AD CSF is removed by Aβ immuno-depletion. **A**: To test whether the seeding activity observed in human AD CSF was dependent on Aβ aggregates, we removed these structures from CSF samples by immuno-depletion using dynabeads coated with a mixture of sequence (4G8) and conformational (A11) antibodies. The procedure was optimized using synthetically prepared Aβ oligomers spiked into human CSF at quantities detectable by western blot. Three-consecutive rounds of incubation with antibody-coated dynabeads were sufficient to efficiently remove the Aβ oligomers observed at around 170 kDa. **B:** Samples of AD CSF before or after immuno-depletion were used to seed Aβ aggregation in the Aβ-PMCA assay. Values represent the average and standard error of 3 different replicates.

Optimization of Aβ-PMCA to detect Aβ oligomers in blood plasma. Detection of Aβ oligomers in blood is much more challenging, likely because the quantity of these species is substantially smaller in blood than in CSF and because blood contains many other components that interfere with the assay. We are developing a strategy to overcome these limitations, which includes the use of ELISA plates coated with various antibodies either specific for the sequence of Aβ or the conformation of Aβ oligomers, to capture Aβ species from plasma and remove other molecules that interfere with the assay. For this purpose, antibody-coated plates were incubated with blood plasma (100 µl). Thereafter, beads are washed and used directly for the Aβ-PMCA reaction. To optimize the assay and estimate the efficiency of detection we used healthy blood plasma spiked with synthetic oligomers. The results showed that we can clearly detect as little as 1 fmol of Aβ oligomers (Fig. 4A). A pilot experiment using a small number of human blood samples from individuals affected by AD, non-AD neurodegenerative diseases (NAD) and healthy controls, showed that the kinetics of Aβ aggregation in the presence of AD plasma was accelerated, and enabled us to distinguish AD from control samples (Fig. 4B). Many more experiments are needed to fully optimize the technology and evaluate its usefulness for detection of Aβ oligomers in human blood.

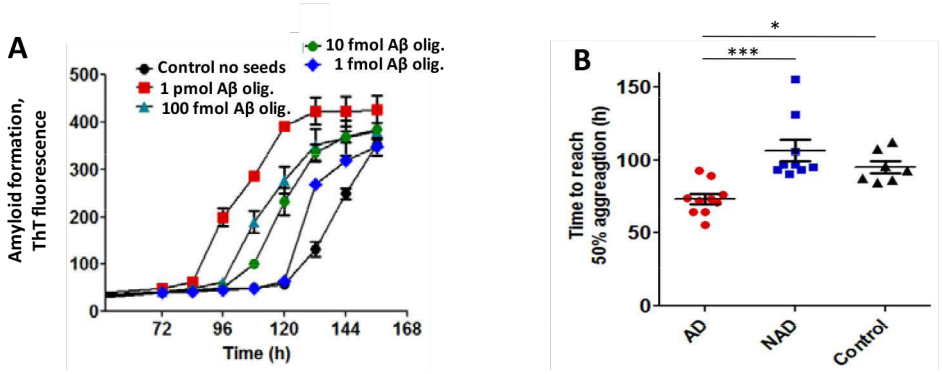

Fig. 4. Detection of seeding-competent Aβ aggregates in human plasma. A: ELISA plates pre-coated with protein G were coated with an anti-conformational antibody. Thereafter, plates were incubated with human blood plasma (100 µl) as such (*control*) or spiked with synthetic Aβ seeds (1, 10, 100 or 1000 fmol of Aβ oligomers). After incubation, plates were washed with PBS-tween and a solution containing containing Aβ40 monomer (2 µM) and ThT (5 µM), prepared in Tris 0.1M, pH 7.4. ThT fluorescence was recorded during the Aβ–PMCA reaction. This figure is representative of several experiments done with 3 different antibodies which work similarly. **B**: Blood plasma samples from patients affected by AD, non-AD neurodegenerative diseases (NAD) and healthy controls were incubated with anti-Aβ antibody (82E1) coated beads and Aβ-PMCA was carried out as discussed in *panel A.* The time needed to reach 50% aggregation was recorded in individual patients in each group. Differences were analyzed by one-way ANOVA followed by the Tukey's post-hoc test. Data in panels A and B cannot be directly compared since plates were coated with different antibodies in each experiment.

RESEARCH DESIGN AND METHODS

Following is a description of the research plan, including milestones and potential pitfalls and alternative approaches. The research plan is separated by the Phase of development, as requested for a STTR fast track application.

PHASE I

Specific Aim 1: Optimization of the experimental conditions for detection of Aβ oligomers in AD blood.

The phase I of this project is designed to optimize assay conditions for the robust, sensitive and specific detection of Aβ oligomers in human blood plasma. The plan is to first optimize the technique using healthy plasma samples spiked with Aβ oligomers experimentally produced *in vitro* from recombinant protein. When reproducible detection of low quantities of synthetic oligomers is achieved, we will switch to work with plasma samples from well-characterized AD patients and transgenic mice models.

Experiment SA1.1. Optimization of Aβ-PMCA for detection of synthetic oligomers spiked in human blood. These studies will follow up the experiments described in preliminary results to optimize the technical details of the assay in order to achieve high reproducibility, sensitivity and specificity. The Aβ-PMCA assay consists of incubating biological fluids containing Aβ oligomers with a small but constant concentration of seed-free recombinant monomeric Aβ, which is used as a substrate to generate long fibrils nucleated by the patients' Aβ oligomeric seeds [8]. The assay combines phases of polymer growth with multiplication of seeds by intermittent shaking, leading to the fragmentation of the polymers and amplification of the seeding process. In the current format the assay is done in 96 well ELISA plates and the read out is the formation of amyloid fibrils which is measured in real time by determining the fluorescence of thioflavin T (ThT). ThT is a die that specifically binds to amyloid fibrillar aggregates and when bound, increases its fluorescence in a manner proportional to the amount of aggregates [49].

One of the main challenges in working with plasma samples, in contrast to our previous work with CSF, is the high complexity of plasma, which contains many other components, some of which interfere with the assay. For this reason, it is necessary to pre-treat the samples with a procedure to enrich Aβ oligomers and remove some of the plasma components that affect the Aβ-PMCA assay. To achieve this goal we have been developing an immuno-affinity based strategy in which Aβ oligomers are separated from other plasma components by a cocktail of sequence-based and conformational antibodies. So far, this has been done using two alternative formats: (i) attaching the antibodies to ELISA plates which will later be used to pull down Aβ oligomers from plasma samples and detect them using the Aβ-PMCA assay. (ii) coupling the antibodies with beads that are incubated with plasma to precipitate Aβ oligomers. Oligomers attached to the beads will be them loaded into ELISA plates where the Aβ-PMCA reaction will be carried out. We will test the following antibodies: 6E10 (recognizes the fragment 1-16 of Aβ), 4G8 (recognizes the fragment 17-24 of Aβ), 82E1 (recognizes the fragment 1-16 of Aβ), A11 (conformational antibody, recognizes misfolded oligomers [48]), and ACU-193 (conformational antibody, recognizes Amyloid beta-Derived Diffusible Ligand [52]).

Synthetic oligomers will be prepared and characterized as described in preliminary results and in our recent publication [8]. Different concentration of synthetic oligomers will be spiked into 2 ml healthy human whole blood (containing EDTA to avoid coagulation), and the plasma fraction will be separated using standard procedures. Thereafter, we will treat the plasma samples with the immuno-affinity enrichment procedure. To optimize the technique, we will test the two assay formats described above and various sequence and conformational antibodies to determine the procedure that most efficiently enriches the oligomers in the sample and best enables their detection by Aβ-PMCA. Controls will include spiking with the same volume of oligomers into buffer only, non-spiked plasma and blood samples spiked with misfolded oligomers composed of non-Aβ proteins, such as α-synuclein and prion protein involved in Parkinson's and prion diseases, respectively. The target sensitivity planned is to be able to detect specifically and reproducibly as little as 1 x 10^{-15} mol of synthetic Aβ oligomers, which corresponds to around 0.1% of total Aβ present in plasma [53]. Since, at this moment the concentration of Aβ oligomers in blood plasma is not known, it might be necessary to further improve this limit of detection. For the procedure to be successful, it is key to have a robust assay that gives very reproducible results.

Experiment SA1.2. Detection of blood endogenous Aβ oligomers by Aβ-PMCA.

When conditions to detect small quantities of synthetic oligomers by seeded cyclic amplification of Aβ misfolding become optimized to the target sensitivity planned, we will attempt to detect putative Aβ oligomers in blood of transgenic animal models and humans diagnosed with probable AD. As negative controls we will use samples

from age-matched non-transgenic mice and from normal people of different ages. For these studies, we will test blood samples from at least 50 transgenic mice collected at a time in which animals have extensive accumulation of amyloid plaques in the brain. We will use transgenic mice over-expressing the human amyloid precursor protein harboring the Swedish mutation (K670M and N671L) and a mutant version of the human presenilin 1 gene ($PSEN1_{\Delta E9}$). As a result, these animals develop amyloid plaques starting at 5-6 months old and abundant plaques are observed in animals older than 9 months of age [54]. A colony of these animals is already available in our animal facility. We will also test plasma samples from at least 50 patients diagnosed with AD in which the diagnosis has been confirmed postmortem by histological analysis of the brain. A similar number of negative controls not affected by neurological disease will be used. For these studies we will use samples already available in our lab or collected from our network of collaborators (see attached letters of collaboration).

Milestones and Timelines for Phase I

1. Determine the best antibodies for immuno-affinity capture of Aβ oligomers and the most efficient assay format to achieve reproducible detection of synthetic Aβ oligomers spiked into human blood. Expected at month 4 of the project.
2. Optimize the experimental conditions for Aβ-PMCA to reproducibly detect at least 1 x 10^{-15} moles of synthetic Aβ oligomers spiked into healthy human blood. Expected at month 7 of the project.
3. Obtain a 95% sensitivity and specificity for detection of Aβ oligomers in transgenic mice plasma. Expected at month 10 of the project.
4. Attain at least 90% sensitivity and 90% specificity for detection of Aβ oligomers in the pilot experiment using human blood samples. Expected at month 12 of the project.

Pitfalls and alternative approaches for experiments in phase I

Our extensive experience with the PMCA technology puts us in a great position to develop this technique for commercial use and to resolve any technical problem we may encounter. However, we may anticipate the following potential pitfalls: **1)** It may be very difficult to obtain a robust amplification assay for detection of Aβ oligomers in blood plasma. Given our experience with the PMCA technology, with Aβ aggregation and the many experiments already performed (some of which are shown in the Preliminary Results), we are certain that it is possible to have good reproducibility in Aβ-PMCA assay under a carefully controlled set of conditions. We have already identified the key technical variables that are essential to achieve good reproducibility. These include the concentration of monomeric Aβ used as substrate, temperature, shaking rate and careful removal of pre-formed aggregates in the recombinant Aβ peptide. **2)** Synthetic Aβ oligomers prepared *in vitro* may not be sufficiently similar to the endogenous Aβ oligomers circulating in blood. Thus, optimization of the technology using synthetic oligomers may not be useful for detection of AD in blood plasma. Considering our recent results in CSF [8], we believe that the aggregates produced *in vitro* mimic reasonably well those formed *in vivo*. In case the Aβ-PMCA works very well in plasma spiked samples, but does not work to detect endogenous Aβ oligomers neither coming from transgenic mice nor from AD patients, we will re-optimize the assay using for spiking Aβ oligomers purified from AD brain. We are currently working on establishing a protocol for high efficiency purification of these aggregates, based on modifications of previously published procedures [55;56]. **3)** Since Aβ oligomers have not been detected in blood plasma before, it is possible that their quantity in this fluid is extremely low or that they may not be present at all. Although, it is possible that Aβ oligomers do not exist in blood, we consider this unlikely, since it is well-known that proteins leak out of the brain, and the quantity of oligomers in this organ in AD patients is likely very high. Indeed, there are several reports showing an equilibrium between the pools of Aβ in brain and blood [57;58]. Also, we have preliminary results showing that blood from transgenic mice models of AD taken at the time in which the animals have substantial amyloid deposits in their brain, is able to induce deposition of Aβ aggregates in young mice, suggesting that blood carries seeding-competent Aβ aggregates (Soto et al., manuscript under review). Although it is unlikely that Aβ oligomers do not exist in blood, it is possible that their quantity in plasma might be very small. However, we believe that with the power of amplification of PMCA, we should be able to detect very tiny amounts of oligomers. Indeed, we have optimized the technology for prion PMCA to detect as little as 1 oligomer of misfolded prion protein in a sample [41]. In case our experiments attempting to detect endogenous Aβ oligomers in plasma of transgenic mice or human beings (Experiments SA1.2) are negative, we will attempt increasing the efficiency of amplification to increase sensitivity of detection.

PHASE II

The main goal of this phase of the project is to perform the studies required for validation and regulatory approval of our technology for a blood-based diagnosis of AD. To achieve this goal, the studies in phase II will be done in direct consultation with the regulatory authorities in the USA and Europe responsible for validation and approval of a diagnostic test for commercialization. The studies will be aimed to investigate the sensitivity, specificity and predictive value of the test using large number of samples (Specific Aim 2) and to evaluate the usefulness of the technology to monitor disease progression and to detect Aβ oligomers in blood before the clinical stage of the disease (Specific Aim 3).

Specific Aim 2. Evaluation of the sensitivity and specificity in large number of plasma samples from AD patients.

We will study the sensitivity and specificity of the technology for detection of Aβ oligomers in blood plasma using large number of samples from people diagnosed with AD and normal controls (both age-matched and young individuals). To further study specificity, we will use samples from people affected by other forms of dementia and neurodegenerative diseases, including Parkinson, Huntington disease, Creutzfeldt-Jakob disease, vascular dementia, fronto-temporal dementia, motor neuron disease, and progressive supranuclear palsy. Finally, we will test samples from patients affected by non-degenerative neurological disorders (including stroke, multiple sclerosis, epilepsy, brain tumors, autoimmune encephalitis, meningitis, etc). The plan will be to include at least 500 AD patients and at least 2000 controls from the various groups listed above. Samples will be provided blinded and only un-blinded at the end of the study.

Because availability of good samples is essential for the success of this project, we have established a network of scientific collaborations with various groups (see enclosed collaboration letters) to obtain large number of well-characterized and carefully collected blood samples from people affected by AD, normal controls of different ages and patients affected by other neurological disorders. The collaborators enlisted in this project are:

A.- Dr [redacted] (co-investigator of this project), Professor of Neurology, Director of the Memory Disorders and Dementia Clinic [redacted] in the University of Texas Medical School at Houston. Dr [redacted] program oversees hundreds of patients at different stages of AD as well as individuals affected by other forms of dementia and neurodegenerative disorders. Patients are well characterized by neuropsychological examination, biochemical measurements of Aβ and Tau in CSF and by recently developed neuroimaging techniques. We already have IRB approval from our institution for collection of blood samples for these studies and, indeed, we have already collected some samples that were used for the preliminary results described in this project.

B.- Dr. [redacted] Director of the Department of Neurodegenerative Diseases and Director of the Division of Neuropathology at [redacted] The [redacted] is an internationally recognized leading centre in neuroscience and belongs to the World Health Organization promoting healthcare. Dr [redacted] has been working on the field of AD and related neurodegenerative disorders for more than 20 years and has already available a large collection of samples. His Unit currently follows many hundreds of patients. We have a long standing collaboration with Dr [redacted] group since more than 10 years ago.

C.- We have approached Dr [redacted] (University of [redacted]), Director of the Biomarker Core at the AD Neuroimaging Initiative (ADNI) to obtain plasma samples from the panel already available at ADNI. ADNI is a public-private partnership founded in 2004 to find more sensitive and accurate methods to detect AD at earlier stages and mark its progress through biomarkers. They have what is likely the best dataset and sample bank available for researchers in the field, which include several hundred plasma samples from AD patients at different stages of the disease, people affected by mild cognitive impairment (MCI) and individuals suffering from diverse neurological diseases.

D.- In collaboration with Dr [redacted] (University of [redacted] we are requesting samples from the [redacted] which is a State funded collaboration between six of the state's leading medical research institutions to improve early diagnosis, treatment, and prevention of AD. The [redacted] repository, of which Dr [redacted] is part, has several hundred plasma samples from AD patients at different stages of the disease, people affected by MCI and normal controls. The database and tissue bank is available to Alzheimer's researchers across the state of Texas.

E.- We will also purchase samples from [redacted] Inc. The [redacted] regulatory-compliant and privacy protected biorepository is the largest private global source of longitudinally collected human samples from normal and diseases populations. Samples are collected through a network of experienced specialist physicians in the US with IRB approved clinical protocols and are provided with detailed clinical data. Sample available

include CSF, blood plasma and urine from the same patients collected longitudinally across different stages of the disease. Currently, the biorepository has samples from over 400 AD patients as well as many hundred samples from individuals affected by diverse diseases and thousands samples from healthy controls.

Specific Aim 3. Study the usefulness of Aβ-PMCA to monitor disease progression and for pre-clinical diagnosis of AD.

The goal of the studies in this Aim is to investigate whether the level of Aβ oligomers in biological fluids (CSF and plasma) correlate with the clinical stage of the disease and the utility of our technology for pre-clinical detection of these particles in blood plasma.

Experiment SA3.1. Correlating plasma Aβ oligomers with the clinical progression of AD

To evaluate whether the levels of Aβ oligomers in CSF and plasma correlate with the clinical progression of AD, we will test samples taken from patients at various stages of AD and we will also perform experiments with longitudinal samples from the same patient collected at different times. Before performing these studies we will attempt to establish a relationship between the concentration of Aβ oligomers present in CSF or plasma and the parameters obtained in the Aβ-PMCA assay. The purpose of this study is to try to use the technology not only to detect Aβ oligomers, but also to estimate their concentration in a biological fluid based on how many cycles of amplification are needed to detect a given quantity of them. The experiments will be done by spiking healthy CSF and plasma with known concentrations of synthetic Aβ oligomers prepared as described in SA1.1 and in preliminary results. After the appropriate pre-treatment, samples will be subjected to cycles of incubation/shaking in the presence of recombinant seed-free monomeric Aβ and ThT as in the Aβ-PMCA protocol and the ThT fluorescence will be recorded automatically every 30 min. When the ThT fluorescence reading is higher than 5-fold the background level, it will be estimated that the aggregation reaction has been initiated and the time will be recorded as the lag phase. The values of the lag phase will be plotted against the concentration of Aβ oligomers spiked in the sample. Based on our experience with prion-PMCA, we expect to obtain a linear relationship between the lag phase and the $\log_{10}$ of the Aβ oligomer concentration. This information will be used to estimate the putative concentration of Aβ oligomers in patient samples. We will assess if we can observe a defined relationship between the concentration of Aβ oligomers and the clinical progression as measured by cognitive scores in samples from AD patients at different clinical stages of the disease and longitudinal samples from the same patient collected at distinct times during the clinical disease. The availability of a biochemical test to monitor disease progression will have important applications for developing drugs for AD treatment and for testing the efficacy of drugs in clinical trials.

As part of the studies included in this aim we also plan to compare the quantity of Aβ oligomers present in the CSF and plasma in a set of patients in which both fluids have been collected at the same time. Our collaborators and the biorepositories from where we will obtain samples, have many samples of CSF and plasma collected at the same time from specific patients. This information will be valuable to further understand the dynamics of Aβ oligomer production, distribution and clearance.

Experiment SA3.2. Evaluate the utility of Aβ-PMCA for pre-clinical diagnosis of AD.

To begin analyzing whether Aβ oligomers detectable by Aβ-PMCA are present in plasma before the onset of amyloid pathology in the brain, we will perform a longitudinal study in a transgenic mice model of AD that spontaneously develops amyloid plaques over time. This experiment will also enable us to estimate the earliest time in which Aβ oligomers can be detected in blood plasma. For these experiments groups of transgenic mice expressing the human amyloid precursor protein harboring the Swedish mutation (Tg2576) will be used. These mice develop amyloid plaques and other neuropathological characteristics of AD starting around 9 months of age and cerebral pathology becomes extensive by 15 months [59]. Blood samples will be taken every other week from a group of control and Tg2576 mice and subjected to Aβ-PMCA for detection of Aβ oligomers.

In order to assess in humans if is possible to detect Aβ oligomers pre-symptomatically by Aβ-PMCA, we will use samples from people with high risk to develop AD, including persons affected by mild cognitive impairment (MCI), asymptomatic carriers of familial AD mutations and Down syndrome individuals. MCI is a particularly interesting option, because a proportion of them naturally progress into AD with time [60;61]. We will measure Aβ oligomers in people that progressed into AD and also in MCI individuals that developed other forms of dementia or remained in the MCI condition. Asymptomatic carriers of mutations associated with familial AD is also an important population to study, since the mutations generally have a high penetrance and the clinical disease develops earlier than in the sporadic form of AD [62]. Down syndrome is a genetic disorder characterized by trisomy of chromosome 21, which is the chromosome where the amyloid precursor protein gene is located. As a result, most (if not all) Down syndrome patients develop dementia and the brain changes

typical of AD at an earlier age [63;64]. Importantly, our collaborators have already available many samples from MCI, carriers of familial AD and Down syndrome in their collection and others will be collected during this project period. The plan will be to test at least 100 samples from MCI patients, 50 carriers of familial AD mutations and 50 individuals affected by Down syndrome of different ages.

Milestones and Timelines for Phase II

1. Obtain at least 90% sensitivity and 90% specificity for detection of Aβ oligomers in the blinded experiment using a large number of human blood samples. Expected at month 32 of the project.
2. Optimize and establish the Aβ-PMCA technology for quantitative detection of Aβ oligomers in blood and CSF through spiking of these fluids with synthetic oligomers. Expected at month 18 of the project.
3. Determine the relationship between the concentration of Aβ oligomers in CSF and plasma and the clinical progression of AD. Expected at month 24 of the project.
4. Analyze the relationship between the quantity of Aβ oligomers present in the CSF and plasma in a set of patients in which both fluids have been collected at the same time. Expected at month 28 of the project.
5. Define the earliest time in which Aβ oligomers can be detected in blood plasma from transgenic mice engineered to develop cerebral amyloid plaques. Expected at month 30 of the project.
6. Determine sensitivity and specificity for detection of Aβ oligomers in plasma samples from pre-clinical AD cases, including individuals affected by MCI, asymptomatic carriers of familial AD and Down syndrome patients. Correlate the levels of plasma Aβ oligomers with the time to first occurrence of clinical symptoms of AD. Expected at month 36 of the project.
7. Regulatory approval of the test. Expected at month 36 of the project.

Pitfalls and alternative approaches for experiments in Phase II

As several of the experiments proposed in this project have already been achieved to some degree (e.g. optimization of Aβ-PMCA for high sensitive detection of Aβ oligomers, detection in human CSF samples, detection in spiked blood plasma, etc), we believe that there is a very good chance that we will be able to reach our ambitious milestones. The experiments in phase II are subjected to some of the same pitfalls as those described for phase I. In addition, we can envision the following potential difficulties: **1)** Since the pathogenesis of AD start years before the onset of clinical symptoms of the disease [65;66], it is likely that a relatively high proportion of our age-matched healthy controls may have Aβ oligomers in blood. We are well aware of this possibility and this is the reason that we will divide our healthy control population in old (age matched subjects, > 55 years of age) and young individuals (<55 years of age). The experiments in SA2 are planned considering a large number of both young and old control samples. In case many of our old (but not young) controls are positive in the Aβ-PMCA test, we will search for samples taken earlier and from whom the complete medical history is available to evaluate which subjects developed AD and at what age. These results may actually be very useful to validate the potential of Aβ-PMCA for early, pre-clinical diagnosis of AD. **2)** Considering the artificial over-expression of mutant genes in transgenic mice, the experiments using plasma samples from these animals may not be informative of the human disease. This is always the case when working with animal models. The experiments planned here using transgenic mice samples are meant only as a proof-of-concept that detection of Aβ oligomers in blood plasma is possible in early stages of the pathogenesis. However, as described in the project we will not rely on these experiments alone for the development and validation of our technology. **3)** It is possible that oligomers formed by other misfolded proteins may seed Aβ aggregation in Aβ-PMCA and result in many false positives in samples from other neurodegenerative diseases. This is certainly a possibility that will be addressed in this project (aim 2) by the experiments with large number of samples from other neurodegenerative brain diseases. In our experience, as well as literature reports, heterologous seeding indeed occurs in some combination of proteins [67], but in general the efficiency of this phenomenon is several orders of magnitude lower than homologous seeding. Therefore, we believe there should be a window of opportunity to detect specifically Aβ over the background of non-specific signal, some of them coming from cross-seeding events. If we find this not to be case, we will attempt to modify the Aβ-PMCA conditions to favor homologous versus heterologous seeding. **4)** There might not be a relationship between the levels of Aβ oligomers in CSF or plasma and the cognitive deterioration. This is entirely possible and more than a pitfall of our technique and project, it will be an interesting result coming from our study. In this case, unfortunately, Aβ-PMCA may not be useful to monitor disease progression and maybe even for pre-clinical identification of individuals on the way to develop AD.

METHODOLOGIES

The team involved in this proposal possesses extensive expertise in all areas needed to successfully complete the project. Dr. Soto and his colleagues and collaborators have considerable knowledge of the PMCA technology, animal modeling of disease, Aβ biology and assay development. We will use methods and protocols developed, standardized and routinely used in our previous studies. For space constrains, below is a description only of the most relevant protocols. For specific methodologies of other procedures we refer the reviewers to some of our recent publications [7;8;10;50;68-74]

Collection of Human Biological samples. CSF and blood samples will be obtained from our network of collaborators and existing biorepositories, including Drs [redacted] Many of the samples needed have already been collected and can be provided rapidly. Others will be collected during the course of the project. The patient clinical data will be carefully collected to achieve the best possible diagnosis and will include all neurological, neuropsychological, imagining and biochemical tests available. For CSF collection, samples will be collected in polypropylene tubes following lumbar puncture at the L4/L5 or L3/L4 interspace with atraumatic needles after one night fasting. The samples will be centrifuged at 3,000 g for 3 min at 4°C, aliquoted and stored at -80°C until analysis. CSF cell counts, glucose and protein concentration will be determined. For blood collection and plasma preparation, we will collect ~10 ml of whole blood into a vacutainer tube containing EDTA. Tubes will be invert several times carefully to mix blood and anticoagulant and thereafter centrifuged at room temperature at 1600 x g for 15 min to separate plasma from the cellular package. Supernatant (plasma) will be carefully aspirated the and deposited in a fresh tube. Aliquots of 0.5 ml will be made in Eppendorf low binding tubes and store at -80 ºC until use. The study will be conducted according to the provisions of the Helsinki Declaration and approved by the respective Ethics Committee.

Collection of Animal Biological Samples. As stated in the research design section, we will use blood plasma samples from two transgenic mice models of AD. Tg2576: This is a widely used mice model of amyloid pathology in AD. These animals express the human APP695 bearing the Swedish mutation (K670N, M671L) and as a result these mice develop amyloid plaques and other neuropathological characteristics of AD starting around 9 months of age [59]. $APP_{Swe}/PSEN1_{\Delta E9}$: A transgenic mice over-expressing the human amyloid precursor protein harboring the Swedish mutation (K670M and N671L) and a mutant version of the human presenilin 1 gene (PSEN1ΔE9). As a result, these animals develop amyloid plaques starting at 5-6 months old [54]. Colonies of these animals are already available in our animal facility. Breeders were purchased from Jackson laboratories. We will obtain mouse blood from retro-orbital sinus by hematocrit glass tube, and this blood sampling method is intended for survival and serial blood samplings during the course of the experiment. The retro-orbital sinus is the site located behind the eye at the medial and lateral canthus. We will anesthetize mice, insert hematocrit glass tube into the sinus and obtain whole blood. For serial blood sampling in mice at various stage of disease progression, the volume withdrawn will be no more than 100 μl (<5 % of total blood volume) at a single bleeding. When larger quantities of blood are needed, we will sacrifice animals. Blood will be collected directly from the heart. Up to 1ml of blood can be collected in this way from each mouse.

Production and purification of Aβ. Aβ peptides will be either chemically synthesized or produced in bacteria. Synthesis will be done at the [redacted] using solid-phase N-tert-butyloxycarbonyl chemistry. Peptide purification will be carried out by reverse-phase HPLC. The final products will be lyophilized and characterized by amino acid analysis and laser desorption mass spectrometry. We have been purchasing our synthetic Aβ peptides from this facility since more than 10 years and it is always very reliable. The production and purification of recombinant Aβ will be done as previously described [75]. Briefly, E. coli cells harboring pET28 GroES-Ub-Aβ42 plasmid will be grown in Luria broth (LB) at 37 °C, and expression will be induced with 0.4 mM IPTG. After 4 h, cells will be harvested and lysed in a lysis buffer (20 mM Tris-Cl, pH 8.0, 10 mM NaCl, 0.1 mM PMSF, 0.1 mM EDTA and 1 mM β-mercaptoethanol) and centrifuged at 18,000 rpm for 30 min. Inclusion bodies will be resuspended in a resuspension buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 1 mM DTT) containing 6 M urea. Insoluble protein will be removed by centrifugation at 18,000 rpm for 30 min. The supernatant containing GroES-Ub-Aβ42 fusion protein will be collected. To cleave off Aβ42 from fusion protein, the fusion protein will be diluted 2-fold with resuspension buffer and treated with recombinant deubiquinating enzyme (Usp2cc) 1:100 enzyme to substrate molar ratio at 37 °C for 2 h. After that, samples will be loaded on a C18 column (25 mm x 250 mm, Grace Vydac, USA). Aβ42 will be purified with a solvent system buffer 1 (30 mM ammonium acetate, pH 10, 2% acetonitrile) and buffer 2 (70% acetonitrile) at a flow rate 10 ml/min using a 20–40% linear gradient of buffer 2 over 35 min. Purified Aβ42 will be lyophilized and stored at -80 °C, until use. This procedure is already established in the lab and the purity and biochemical characteristics of the peptide have been extensively studied.

Preparation of seed-free Aβ for Aβ-PMCA substrate. Seed-free monomeric Aβ will be obtained by size exclusion chromatography, as described [76]. Briefly, an aliquot of a 1 mg/ml peptide solution prepared in dimethylsulfoxide will be fractionated using a Superdex 75 column eluted at 0.5 ml/min with 10 mM sodium phosphate, pH 7.5. Peaks will be detected by UV absorbance at 220 nm. The peak corresponding to 4-10 kDa molecular weight containing monomer/dimmers of Aβ will be collected and concentration determined by amino acid analysis. Samples will be stored lyophilized at -80C.

Preparation of synthetic Aβ oligomers. To prepare different types of aggregates, solutions of seed-free Aβ1-42 (10 μM) will be incubated for different times at 25 ºC in 0.1 M Tris-HCl, pH 7.4 with agitation. This preparation contains a mixture of Aβ monomers as well as fibrils, protofibrils and soluble oligomers in distinct proportions depending on the incubation time. The degree of aggregation will be characterized by ThT fluorescence emission, electron microscopy after negative staining, dot blot studies with the A11 conformational antibody [48] and western blot after gel electrophoresis using the 4G8 anti-Aβ antibody.

Cyclic Amplification of Aβ misfolding and aggregation (Aβ-PMCA). Solutions of 2 μM aggregate-free Aβ1-42 in 0.1 M Tris-HCl pH 7.4 (200 μl total volume) will be placed in opaque 96-wells plates and incubated alone or in the presence of synthetic Aβ aggregates or patients samples (CSF or blood plasma), pre-treated as will be defined during this project. Samples will be incubated in the presence of 5 μM Thioflavin T (ThT) and subjected to cyclic agitation (1 min at 500 rpm followed by 29 min without shaking) using an Eppendorf thermomixer, at a constant temperature of 22 ºC. At various time points, ThT fluorescence will be measured in the plates at 485 nm after excitation at 435 nm using a plate spectrofluorometer. For some of the experiments, we will utilize the FLUOstar OPTIMA microplate reader that enables to perform the cycles of incubation and shaking and measure automatically the ThT fluorescence emission during the entire course of the experiment without manual intervention. This machine has been successfully used to cyclically amplify misfolded prion protein implicated in prion diseases [77].

Determination of Sensitivity, Specificity and Predictive Value. The differences in the kinetic of aggregation between different samples will be evaluated by the estimation of various different kinetic parameters, including the lag phase, the A50 and P90. Lag phase is defined as the time required to reach a ThT fluorescence which is higher than 5 times the background value of the buffer alone. The A50 corresponds to the time in which it is reached 50% of the maximum aggregation. P90 corresponds to the extent of aggregation (measured as ThT fluorescence) at 90 h. Sensitivity, specificity and predictive value will be determined using this data, with cutoff thresholds determined by Receiver Operating Characteristics (ROC) curve analysis, using the MedCalc software (version 12.2.1.0).

Statistical analysis. The significance of the differences in Aβ aggregation kinetics in the presence of human CSF samples will be analyzed by one-way ANOVA, followed by the Tukey's multiple comparison post-test. The level of significance was set at $P<0.05$. Statistical tests will be performed using the Graph Pad Prism 5.0 software.

Human subjects

This project does not involve research on human subjects, since no intervention or interaction with living patients is involved and no identifiable data would be disclosed for this research. Some of the blood samples needed are also already available in our lab, however additional samples of blood will be obtained from our Alzheimer's clinic directed by Dr[redacted]. We already have an IRB approval for the collection of these samples. Additional samples will be obtained de-identified from our network of collaborators and existing biorepositories , including [redacted]

1. Human Subjects Involvement and Characteristics:

a. Describe the proposed involvement of human subjects in the work outlined in the Research Design and Methods section.

The studies involve the use of blood samples from patients affected by AD and other neurological disorders as well as normal individuals of different ages. Biochemical diagnosis of these diseases is a high unmet medical need to prevent further spreading of the disease.

b. Describe the characteristics of the subject population, including their anticipated number, age range, and health status. If the characteristics of the population are not available, then the applicant should indicate that the information is unknown.

We will use blood samples from individuals affected by AD, normal controls from different ages and samples from people affected by other neurological conditions, including diverse forms of dementia and neurodegenerative diseases (such as Parkinson, Huntington disease, Creutzfeldt-Jakob disease, vascular dementia, fronto-temporal dementia, motor neuron disease, and progressive supranuclear palsy) and well as non-degenerative neurological disorders (including stroke, multiple sclerosis, epilepsy, brain tumors, autoimmune encephalitis, meningitis, etc).
The estimation is that for our complete set of studies we will need samples of blood plasma (1 ml) from around 700 AD patients and an additional 2200 controls and people affected by other neurological disorders (the total distributed among the diseases listed above). We will also need samples from people affected by mild cognitive impairment (MCI), asymptomatic carriers of familial AD mutations and persons with Down syndrome of different ages.

c. Identify the criteria for inclusion or exclusion of any subpopulation.

The criteria will be purely based on clinical examination. We do not include or exclude people on the bases of sex, race or any other reason than the presence of the disease.

d. Explain the rationale for the involvement of vulnerable populations, such as fetuses, neonates, pregnant women, children, institutionalized individuals, or others who may be considered vulnerable populations. Exemptions 1-6 do not apply to research involving prisoners or subjects who become prisoners (see 45 CFR Part 46 Subpart C). Although Exemptions 1 and 3-6 apply to research involving children (see 45 CFR Part 46 Subpart D), Exemption 2 can only be used for research involving observations of public behavior when the investigator(s) do not participate in the activities being observed.

There is no involvement of vulnerable populations, except for people suffering from Down syndrome. As explained in the project, Down syndrome patients have 3 copies of chromosome 21, which is the chromosome where the amyloid precursor protein gene is located. As a result, most (if not all) Down syndrome patients develop dementia and the brain changes typical of AD at an earlier age. These samples will be used to analyze the predictive power of our test.

e. List any collaborating sites where human subjects research will be performed and describe the role of those sites in performing the proposed research.

The samples will be obtained from a network of collaborators as listed before.

2. Sources of Materials:

a. Describe the research material obtained from living human subjects in the form of specimens, records, or data.

The material will consist of 1ml of blood plasma from each patient.

b. Describe any data that will be recorded on the human subjects involved in the project.

The data will not be associated to any personal information from the patient. Samples will be blindly codified and no personal information will be available to us other than the disease clinical features.

c. Describe the linkages to subjects, and indicate who will have access to subject identities.

Vertebrate Animals

1. Provide a detailed description of the proposed use of the animals in the work outlined in the Research Design and Methods section. Identify the species, strains, ages, sex, and numbers of animals to be used in the proposed work.

We will use two different transgenic models of AD in mice. Mice expressing the human mutant APP gene (KM670/671NL) or the mutant APP plus PS1 have been acquired from Taconic and the colonies have been established in our lab. These animals develop many of the pathological features of AD, including extensive deposition of amyloid plaques starting at 6 or 12 months of age (for single and double transgenics), neuritic dystrophy, astrogliosis and behavioral alterations. We estimate that during the course of this study we will use approximately 200 animals.
The use of animals is mostly to obtain blood samples. We will obtain mouse blood from retro-orbital sinus by hematocrit glass tube, and this blood sampling method is intended for survival and serial blood samplings during the course of the experiment. The retro-orbital sinus is the site located behind the eye at the medial and lateral canthus. We will anesthetize mice, insert hematocrit glass tube into the sinus and obtain whole blood. For serial blood sampling in mice at various stage of disease progression, the volume withdrawn will be no more than 100 μl (<5 % of total blood volume) at a single bleeding. When larger quantities of blood are needed, we will sacrifice animals. Blood will be collected directly from the heart. Up to 1ml of blood can be collected in this way from each mouse.
These techniques are already implemented and routinely done in Dr Soto's lab as part of the Drug Discovery and Development Unit within the Mitchell Center for Alzheimer's disease and related Brain Disorders.

2. Justify the use of animals, the choice of species, and the numbers to be used. If animals are in short supply, costly, or to be used in large numbers, provide an additional rationale for their selection and numbers.

Transgenic mice expressing the human mutant Amyloid precursor protein gene alone or in conjunction with the presenilin 1 gene are the best available animal models to study accumulation and deposition of Aβ aggregates. These animals develop in an age-dependent way many of the neuropathological, biochemical and clinical alterations observed in AD, including amyloid deposition, neuritic dystrophy, astrogliosis, synaptic alterations and behavioral abnormalities. The number of animals to be used has been reduced to the minimum to obtain the needed quantities of samples.

3. Provide information on the veterinary care of the animals involved.

Transgenic mice will be housed in the animal facility located in the Medical School Extension building. This is a state-of-the-art, recently built facility that is operated in accordance with the AAALAC guidelines. The vivarium is maintained under strict supervision by an animal caretaker and includes permanent veterinary care as needed. The Animal Welfare Committee is responsible for University-wide oversight of compliance with all applicable laws, regulations, and policies relating to the humane care and appropriate use of animals in teaching, testing, and research.

4. Describe the procedures for ensuring that discomfort, distress, pain, and injury will be limited to that which is unavoidable in the conduct of scientifically sound research. Describe the use of analgesic, anesthetic, and tranquilizing drugs and/or comfortable restraining devices, where appropriate, to minimize discomfort, distress, pain, and injury.

We do not expect that the procedures for biological fluid collection will be painful, since are performed under anesthesia. Animal recovery after anesthesia will be carefully monitored for signs of any severe abnormality (such as seizure, convulsion, locomotor impairment, extreme vocalization, substantial loss of weight or coma). In case any of these symptoms are observed, the animals will be humanely euthanized to avoid further pain. If there is evidence for slight pain or distress we will consider to provide some analgesic treatment upon consultation with the veterinary in the animal facility.

5. Describe any method of euthanasia to be used and the reasons for its selection. State whether this method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. If not, present a justification for not following the recommendations.

Mice will be humanely killed by asphyxiation with CO_2. Compressed CO_2 from cylinders is the only acceptable source. Mice will be introduced into a top-opening chamber. After the animal(s) are placed in the chamber, a slow flow of CO_2will be initiated for a few minutes to slowly establish a high concentration at the bottom of the chamber. After breathing has stopped and the animal(s) are unconscious, euthanasia will be completed by continued exposure to CO2 for 5-30 minutes after breathing has stopped. Death will be further confirmed by cervical dislocation.

Governance and Organizational Structure:
Dr. Vollrath and Soto will be responsible for leading this project, according to their individual expertise, as described above. They will assure the implementation of the Scientific Agenda, the Leadership Plan, and the Specific Aims, and ensure that systems are in place to guarantee institutional compliance with US laws, DHHS, and NIH policies including biosafety, human and animal research, data and facilities.

Administrative, Technical, and Scientific Responsibilities:
Dr. Vollrath will assume all administrative management and reporting duties for communication with the NIH and submission of annual reports (which will be prepared together with Dr. Soto).

Data Sharing:
Drs. Vollrath and Soto will freely share data and provide access to each other's laboratories for all the members of this project with the goal to foster communication. Powerpoint presentations of the data presented during joint meetings will be made available to all members of the two research groups.
Publication and authorships will be based on the relative scientific contributions of the PIs and their research personnel.

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