Project Title: Study for the early detection of cardiovascular damage in Chagas disease. Development of a new non-invasive diagnostic test based on an specific micro RNA pattern identification

I. Project Purpose and Background

Brief overview of prior work leading to the project: The main goal of their research and interventional actions were the prevention of sudden death among young asymptomatic patients infected with *Trypanosoma cruzi (T. cruzi),* and included the epidemiological surveillance of rural and urbanized regions, population risk stratification, analysis of cardiac rhythm disturbances, evaluation of different therapeutic strategies, a very extensive educational program for the community at risk -and also for physicians- directed towards the improvement of disease detection and control, and sampling of antibodies against muscarinic receptors in different Latin American centers . However, up to the present, worldwide research had no major impact on both disease control and risk reduction of disease complications. Taking advantage of recent molecular biology discoveries, we decided to focus on prevention of cardiovascular complications in an early phase of Chagas´ disease evolution, i.e. the "indeterminate phase" defined by serological confirmation of active *T. cruzi* infection, but without clinical manifestations or cardiovascular alterations in traditional complementary tests. The aim of this proposal is to identify surrogate cardiovascular damage biomarkers that will allow to detect those young asymptomatic chagasic patients that have an increased risk of sudden death or discapacity, in order to offer them the chance of receiving protective cardiovascular treatment.

Global impact of the project: is to reduce morbidity and sudden death in indeterminate phase Chagas´patients. To achieve this goal we propose to search for a minimally invasive and low cost diagnostic method that can be applied in economically troubled Latin American public hospitals, and worldwide, for the early detection of subclinical cardiovascular damage in this patient population. If the project is successful it would provide a new tool not only for diagnosis but also for intervention of asymptomatic patients, since treatment is currently indicated when damage is more advanced, clinical changes are present and can be detected in complementary studies.
Background. A) *General Aspects of Chagas´disease*. Chagas` disease, caused by the protozoan *T cruzi*, is one of the main medical problems and fourth leading cause of mortality in Latin America, affecting 16 to 18 million people. A wide part of infected individuals will become handicapped and/or die prematurely, in general due to the cardiac complications of the disease. As a result of migration, Chagas´ disease has spread from endemic to developed countries –mainly the United States, Canada, Australia, and Spain. *T. Cruzi* is transmitted to humans by blood feeding insects (family Reduviidae, subfamily Triatominae) that hide in crevices inside human dwellings. For persons in the acute phase the death rate is ~10% due to myocarditis or meningoencephalitis. In most patients, the initial infection occurs unnoticed at a very young age, and they may pass to the chronic stage of the disease several decades later. In consequence, those individuals that become handicapped and/or unfit to work by the complications and consequences of Chagas´ disease range between the ages of 30 and 40 years old. B) *The indeterminate phase of Chagas disease: an opportunity for interventional prevention*. After the primary infection, >95% untreated acute patients enter an “indeterminate” phase characterized by the

presence of infection, in the absence of symptoms or detectable cardiac abnormalities. However, necropsy studies revealed that even when no symptoms are evident, cardiac changes occur at an early stage and seem to be secondary to microvascular disease. About half of indeterminate phase patients will silently evolve to a chronic phase and progress to severe cardiac damage. Currently, there are no vaccines for prevention of the disease. Chagas´ disease patients belong to low income groups, and are mostly detected when they attend public hospitals -once they already present cardiac symptoms- during consultation for other reasons, such as pregnancy. Most Latin American public hospitals lack the economic resources and complex diagnostic image infrastructure that would allow the early detection of subclinical cardiac damage. Due to the lack of symptoms no therapeutic measures are taken to protect the heart in indeterminate chagasic patients. If the present proposal is successful, it will be possible to recognize those indeterminate chagasic patiens that are at risk of progressing to severe Chagas heart disease, and to take preventive steps to retard/prevent fatal or incapacitating outcomes.

C) *Long term goals*. i) To set the bases for the development of safe, effective and economically feasible drug or biotechnology (miRNA mimics or antagonists) treatments for cardiovascular protection in indeterminate chagasic patients, ii) to raise awareness of public health authorities on the need to take measures to prevent/attenuate the progression to discapacity in chagasic patients, iii) to advocate for public health support to deal with diseases that do not receive enough attention in this early phase.

II. Project Framework

Project Overview	Indicators of Success	Monitoring & Evaluation
<u>Strategic Area</u>: Neglected and other infectious diseases	An increase in the number of early phase (indeterminate phase) Chagas´ disease patients that are diagnosed as having subclinical cardio-vascular damage	
<u>Project Goal</u>: The final impact of the project is to reduce morbidity and sudden death in early phase of Chagas´disease (indeterminate phase)	An increase in the number of indeterminate chagasic patients that are diagnosed with clinically undetectable cardiovascular damage, and are submitted to protective cardiovascular treatment.	Data will be collected from public Hospitals in Argentina.

Objectives: 1. To assess the prevalence of early minimal (clinically undetectable) cardiovascular damage in early (indeterminate) chagasic patients	1. Obtention of a report showing the prevalence of early minimal cardiovascular damage in a population of indeterminate chagasic patients from Argentinean hospitals.	1. Subclinical cardiovascular status will be assessed by use of gadolinium-enhanced cardiovascular magnetic resonance, echocardiogram, arterial Doppler ultrasound, and 64-slice carotid tomography.

<u>Objectives</u> (cont) 2. To identify a minimally invasive biochemical indicator as a surrogate marker of early minimal (clinically undetectable) cardiovascular damage in indeterminate Chagas ´patients	2. The identification of a group of ~40 specific blood plasma microRNAs that are either over- or underexpressed in those indeterminate Chagas´ patients that present minimal (clinically undetectable) heart/ vascular damage, as compared with microRNA levels in healthy individuals and Chagas´ patients without minimal cardiovascular damage.	2. Blood plasma obtained from indeterminate chagasic patients derived from Buenos Aires city public hospitals will be used to analyze the expression of 875 plasma microRNAs (microarray), We expect to identify ~40 microRNAs showing either <0.5- or >1.5-fold changes versus baseline expression in healthy individuals and Chagas´ patients without minimal cardiovascular damage.
3. To provide economically limited public hospitals with a screening test to identify indeterminate Chagasic patients at risk of progressing to incapacitating cardio-vascular complications and/or sudden death	3. Obtention of a new low cost and minimally invasive cardiovascular status screening method for indeterminate Chagasic patients.	3. From the initial ~40 microRNA set we expect to optimize the detection by Northern blot or polymerase chain reaction (two techniques available in public hospitals) of a 5-microRNA subset with diagnostic value for minimal heart/vascular damage in indeterminate Chagas´patients

III. General Approach. Indeterminate chagasic patients -defined as asymptomatic individuals that are seropositive for *T cruzi* infection, without clinical evidence of abnormalities, with normal electrocardiogram, echocardiogram and chest radiograph- derived from Buenos Aires city public hospitals will be selected. INCLUSION CRITERIA: male or female, ages 18-45 years, with *T. cruzi* infection confirmed by indirect immunofluorescence, indirect hemagglutination, and/or enzyme-linked immunosorbent assay. Patients positive by at least 2 tests will be identified as seropositive. EXCLUSION CRITERIA: presence of cardiovascular risk factors, including hypertension, diabetes, dyslipemia, obesity, smoking habits, alcoholism, and clinically significant renal disease; endocrinopathy, or any other disease with cardiac involvement. A written informed consent will be obtained from all patients and the study conducted in accordance with the Principles of Good Clinical Practice and Declaration of Helsinki.The protocol was approved by the Austral University Hospital Research Ethics Committee. PATIENT GROUPS: To investigate cardiovascular status, 60 to 100 consecutive indeterminate chagasic patients will be submitted to cardiovascular studies in order to identify 20 patients with, and 20 patients without, subclinical cardiovascular damage (see indicators below). Twenty age and sex matched healthy individuals will be submitted to the same studies to confirm the absence of subclinical cardiovascular damage. SUBCLINCAL CARDIOVASCULAR DAMAGE IDENTIFICATION: a) myocardial fibrosis by gadolinium-enhanced cardiovascular magnetic resonance and backscattering tisular echocardiogram ; b) functional and structural vascular damage by assessing endothelial function (brachial artery endothelial-dependent flow-mediated vasodilation), carotid artery intima-media thickness, and pulse wave velocity with a non invasive Vivid 5 echodoppler high resolution vascular scanner (GE Health Care) with a linear array 10 mHz transducer, with images being processed with an Hemdoyn 4 (DINAP SRL, Argentina), c) 64-slice carotid tomography. IDENTIFICATION OF A DIAGNOSTIC SET OF PLASMA MICRORNAs. Blood samples will be collected, and plasma used for

microarray analysis of the expression of 875 human microRNAs. Real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) will be used as the gold standard for validation of microarray expression data. Full data analysis will be conducted by using the services of LC Sciences. We expect that an ideal marker will be expressed in the heart and/or vascular cells at moderate or high levels and will be present at very low or undetectable levels in plasma from healthy individuals. We created a list of likely blood-based miRNA biomarker candidates for subclinical heart damage by compiling a list of miRNAs expressed in human/rodent cardiovascular pathology specimens based on published miRNA expression profiling data and by filtering out miRNAs detected in healthy donor-derived plasma in published work. Detection of blood plasma microRNAs by Northern blot or PCR will be conducted by optimization of the standard technologies. Receiver Operating Characteristic analysis will be used to assess the sensitivity and specificity of Northern blot- or PCR-based identification of the putative microRNA subset for the detection of subclinical cardiovascular damage.

IV. Major Assumptions a) In politically complex Argentina, the economical instability may retard the normal development of the project. However, the prestige and economic solvency of the institution may allow to circumvent this potential problem; b) If we are unable to detect indeterminate chagasic patients without minimal cardiovascular damage, i.e. all indeterminate chagasic patients present subclinical cardiovascular damage, the group of healthy individuals will serve as a control against which the expression of the microRNA set will be compared to assess its diagnostic value for subclinical cardiovascular damage.

V. Estimated Budget by Objective

Objective	Year 1	Year 2	Year 3	Year 4	Year 5	Total
Objective 1	105,000					
Objective 2	30,600					
Objective 3		58,900				
Total	135,600	58,900				

VI. Organizational Experience and Collaborative Partnerships The Austral University (AU) understands that the search for truth through scientific research is an essential part of its mission. The School of Biomedical Science at the AU (SBMS-AU) created in 1999 the Direction of Research, with the objective of promoting the advancement of scientific research, coordinating the activities of the different academic units and facilitating the relations with public and private institutions. A relevant characteristic of the SBMS-AU is the easy concretion of research initiatives due to the presence of a structure composed of a Clinical Research Unit, a Committee for the Evaluation of Research Studies, an independent Ethics Committee, and advanced technical resources that add up to the excellence of the Institution. Regarding the Institutional experience on the assessment of cardiovascular status, the AU Hospital counts with an Arterial Hypertension Center -directed by Dr Carol Kotliar- that specializes in the non-invasive evaluation of blood vessels, and conducts approximately 200 studies per month. The Hospital General de Agudos Dr. Teodoro Alvarez has ample experience in the care and management of chagasic patients.