Role of serum biomarkers in prediction and prognosis of Chagas' disease

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1. Introduction

1.1. Cardiac disease - an overview

Cardiac disease or cardiopathy is a broad term for a variety of diseases affecting the heart. In modern times, cardiac disease has emerged as the leading cause of death worldwide, particularly in developed countries. According to the World Health Organization (WHO), 16.7 million deaths worldwide in 2003 (29.2% of total global deaths) were caused by some form of cardiovascular disease. Though the rate of cardiac disease is highest in developed countries, developing countries are also seeing an increase in occurrence of cardiac diseases, as well as corresponding rise in the number of heart-related deaths (Ahrens I *et al*., 2010). The WHO estimates that by 2010, cardiac disease will surpass Acquired immune deficiency syndrome (AIDS) as the leading cause of death in developing countries (WHO 2006).

The resulting costs of cardiac disease are diverse, starting from the cost to individual and his family of health care and time off work, the cost to the government of health care, and finally the cost to the country owing to lost productivity. According to the American Heart Association, the cost of cardiovascular diseases and stroke in the United States was estimated to be $503.2 billion in 2010, which is higher than for any other diagnostic group (Lloyd-Jones D *et al*., 2010). This included both the direct and indirect costs. The direct costs included the cost of physicians and other health professionals, hospital and nursing home services, the cost of medications, home health care and other medical durables. Indirect costs include lost productivity that results from illness and death. This is only the economic cost. The true cost in human terms of suffering and lost lives is incalculable.

1.1.1. Heart failure– a rising global burden

Heart failure (HF) is generally defined as inability of the heart to supply sufficient blood flow to meet the body's need. The common causes of HF include myocardial infarction (heart attack) and other forms of ischemic heart disease, hypertension, valvular heart disease, and cardiomyopathy (McMurray and Pfeffer, 2005).

HF is a common, costly, disabling, and potentially deadly condition. In developed countries, around 2% of adults suffer from HF, but in those over the age of 65, this increases to 6-10% (McMurray and Pfeffer, 2005; Dickstein K *et al*., 2008). It is associated with high health expenditure mostly due to hospitalization costs. In the United Kingdom, these costs account for almost 2% of the total budget of the National Health

Service, while in the United States it amounts to more than $35 billion (Stewart S *et al*., 2002; Rosamond W *et al*., 2008). HF is associated with significantly reduced physical and mental health, resulting in a marked decrease in quality of life (Juenger J *et al*., 2002; Hobbs FD *et al*., 2002). With the exception of HF caused by reversible causes, the condition usually worsens with time. Although some people survive many years, progressive disease is associated with an overall annual mortality rate of 10% (Neubauer S, 2007). It is the leading cause of hospitalization in people older than 65 years (Krumholz HM *et al*., 2000). HF affects approximately 5 million people in the United States, and each year close to 500,000 new cases are diagnosed. The number of deaths in the United States from this condition has more than doubled since 1979, averaging 250,000 annually (Allman E *et al*., 2009).

1.1.2. What is dilated cardiomyopathy?

Cardiomyopathy which literally means "heart muscle disease" is the impairment of function of the myocardium (i.e. the actual heart muscle), due to any reason. In practice, this term is reserved for severe myocardial disease leading to HF. People with cardiomyopathy are often at risk of arrhythmia or sudden cardiac death or both (Kasper DL *et al*., 2005).

Dilated cardiomyopathy (DCM) is a type of cardiomyopathy in which heart muscle becomes weakened and enlarged, and cannot pump blood efficiently anymore. The decrease in heart function can affect the lungs, brain, liver and other body systems. In DCM, a portion of the myocardium is dilated, often without any obvious cause. Left or right ventricular systolic pump function of the heart is impaired, leading to progressive cardiac enlargement and hypertrophy by the process of remodeling.

DCM is the most common form of non-ischemic cardiomyopathy. It occurs more frequently in men than in women, and is most common between the ages of 20 and 60 years. About one in three cases of congestive heart failure is due to dilated cardiomyopathy (Robbins SL *et al*., 2003; Kasper DL *et al*., 2005).

Although in many cases no cause (etiology) is apparent, DCM is probably the result of damage to myocardium produced by a variety of toxic, metabolic, or infectious agents. It may be due to a fibrous change of the myocardium resulting from a previous myocardial infarction. Acute viral myocarditis such as with coxsackievirus B and other enteroviruses, may also present with DCM as a late sequel, possibly being mediated via an immunological mechanism (Martino TA *et al*., 1994). Autoimmune mechanisms are also

suggested as a cause for DCM (San Martin MA *et al*., 2002). However, many cases of DCM are described as idiopathic - meaning that the cause is unknown.

1.1.3. Chagas' disease

1.1.3.1. Background of Chagas' disease

Chagas' disease (CD) (also known as American trypanosomiasis) is a parasitic disease caused by the hemoflagellate protozoan, *Trypanosoma cruzi* (*T.cruzi*). It is found mainly in Central and South America, where it is transmitted to humans and other mammals mostly by an insect vector, the blood sucking bugs of the subfamily Triatominae (family Reduviidae). Nearly 10 million people are infected worldwide, and more than 25 million people are at risk of the disease (Chagas disease [American trypanosomiasis] fact sheet [revised in June 2010]).

Findings from paleoparasitology studies on *T.cruzi* DNA recovered from human mummies have revealed that CD afflicted man as early as 9000 years ago (Aufderheidi AC *et al*., 2004). However, it was not until 1909 when a Brazilian physician, Carlos Chagas (1879-1934) described the disease for the first time, which was later named after him. Chagas' work is unique in history of medicine because he was the only researcher so far to describe solely and completely a new infectious disease: its pathogen, vector, host, clinical manifestations, and epidemiology. Infact, the first reported case of CD might have preceded Carlos Chagas' discovery- Charles Darwin quite possibly contracted *T.cruzi* infection during his expedition to South America in 1835, as suggested by his vivid description of contact with the kissing bug, triatomine, and by some of his symptoms in later life (Bernstein RE, 1984).

1.1.3.2. Pathogenesis of Chagas' disease

In acute phase of *T.cruzi* infection, organ and tissue damage is caused by the parasite itself and by the acute inflammatory response of the host, which is induced by the presence of the parasite (Andrade ZA, 1999). There is a strong T-helper-1 immune response with presence of both CD4 and CD8 cells, and characterized by the production of certain cytokines like tumour necrosis factor alpha (TNF alpha), interferon gamma, and interleukin 12 (IL-12), that are important in the control of parasitism (Abrahamsohn IA and Coffman RL, 1996; Silva JS *et al*., 1998; Martins GA *et al*., 1999). These cytokines cause the activation of trypanocidal activity of human macrophages mainly via a nitric oxide (NO)-dependent mechanism (Chandra M *et al*., 2002; Gutierrez FR *et al*., 2009). In

comparison, production of cytokines like IL-10 and transforming growth factor beta (TGF beta) lead to increased replication and resistance of the parasite due to inhibition of macrophage trypanocidal activity (Silva JS *et al*., 1991; Reed SG *et al*., 1994). Overall the T-helper-1 immune response has a protective role against *T.cruzi* infection mainly through the synthesis of nitric oxide that exerts a potent trypanocidal action.

During chronic infection, the balance between immune mediated parasitic control and inflammatory damage to host tissues probably determines the course of disease. In case, the immunological response is inefficient and causes tissue damage, the parasite load will increase and further exacerbate the immune-mediated inflammation. In contrast, a well mediated immune response will not only lead to effective parasitic control, but also minimize the damaging effects of inflammation on host tissues (Tarleton RL, 2003).

Although the pathogenesis of chronic CD is not well understood, there is a growing consensus that the persistence of parasite inside the host tissue is necessary for development of disease (Kierszenbaum F, 2007; Bonney KM and Engman DM, 2008). However, it is still unclear whether tissue damage mostly occurs due to direct parasite factors, or is indirectly caused by parasite-induced immunopathological or auto-immune mechanisms (Soares MB *et al*., 2001; Marin-Neto JA *et al*., 2007).

In chronic CD patients with cardiomyopathy, there is low intensity but slowly progressive, persistent myocarditis that leads to gradual impairment of contractile function and dilation of all four chambers of the heart. Left ventricular apical aneurysms, thromboembolic disease and other segmental wall motion abnormalities are common in the relatively early stages of the disease (Acquatella H, 2007). Histological examination reveals widespread destruction of myocardial cells, edema, diffuse fibrosis, mononuclear cellular infiltration of the myocardium and scarring of the conduction system (Andrade ZA, 1983). All these changes are responsible for the frequent pathological occurrences of atrio-ventricular blocks, inter-ventricular blocks, and sinus nodal rhythm dysfunction in CD. Progressive loss of myocardial fibers and the replacement of dead myocytes by intense fibrosis predispose the patient to development of HF and ventricular arrhythmias (Rassi A Jr *et al*., 2000; Rassi A Jr *et al*., 2009). In addition, pathological changes in coronary microvasculature may further exacerbate the myocardial damage (Rossi MA, 1990; Marin-Neto JA *et al*., 1992).

Chronic Chagas' gastrointestinal disease is caused by destruction of intramural autonomic ganglia, predominantly in the esophagus, colon or both. This leads to loss of peristaltic

movements in the affected sections of the gastrointestinal tract, thus causing mega disease (megaesophagus and/or megacolon) (Kőberle F, 1968).

1.1.3.3.   Clinical manifestations of Chagas' disease

Human exposure to *T.cruzi* may lead to an acute Chagas' infection, which usually lasts for 4-8 weeks. The chronic phase that follows persists for lifespan of the host (WHO, 2002; Rassi A Jr *et al*., 2010). Acute phase is in most cases asymptomatic, but sometimes it may present as a self-limiting febrile illness. Symptoms usually occur 1-2 weeks after exposure to the infected triatomine bugs, or up to a few months after infected blood transfusion. Treatment with anti-parasitic drugs like benznidazole cures most cases of acute infection (Pinto AY *et al*., 2009), and prevents any chronic manifestations. However, death can occasionally occur in acute phase (<5-10% of symptomatic cases) due to severe myocarditis or meningoencephalitis, or both.

Symptoms of acute phase subside spontaneously in about 90% of patients even without initiating anti-trypanosomal treatment. Almost 60-70% of infected individuals will then enter an asymptomatic phase, where they will never develop any clinically apparent disease. Such patients have the indeterminate form of chronic CD, which is characterized by positivity for anti-*T.cruzi* antibodies in patient's serum, a normal 12-lead electrocardiogram (ECG), and normal radiological scans of the chest, esophagus, and colon. The remaining 30-40% of cases will develop a determinate (clinical) form of chronic CD, usually 10-30 years after the initial infection (Pinto Dias JC, 1995). The clinical manifestations may be in the form of cardiac, digestive (megaesophagus and megacolon), or cardiodigestive diseases. A direct progression from acute phase to a clinical form of CD may occur in few patients (5-10%). Reactivation of CD is also possible in chronically infected patients who develop an immunocompromised condition due to immunosuppressive drug therapy, or co-infection with human immunodeficiency virus (HIV) (LM Braz *et al*., 2008).

1.1.3.3.1. Acute Phase

The acute phase of CD is mostly asymptomatic, which is probably due to the low parasite load. When present, symptoms usually include fever, headache, malaise, and mild enlargement of spleen, liver, and lymph nodes. There may be clinical findings at the site of inoculation of *T.cruzi*, either in the eye_ Romaña sign (unilateral eyelid edema, conjunctivitis, and lymphadenopathy), or on the skin_ a chagoma (firm, dusky red swelling with localized lymphadenopathy). An ECG might reveal sinus tachycardia, low

QRS voltage, first-degree atrioventricular block, or primary T-wave changes. There may also be variable degrees of cardiomegaly visible on a chest radiograph (Rassi A Jr *et al*., 2000; WHO 2002).

In congenitally infected infants, symptoms usually appear at birth or few weeks after delivery. Common symptoms include fever, hypotonicity, hepatosplenomegaly, and anemia. There may be findings of prematurity and low birth-weight (Bittencourt AL, 1976; Freilij H and Altcheh J, 1995). In-utero infections can also lead to placentitis and abortion. Serious manifestations like meningoencephalitis and myocarditis are rare in CD patients, but when present, they have high risk of mortality (Torrico F *et al*., 2004).

1.1.3.3.2. Chronic Phase

The typical clinical manifestations of chronic phase are related to pathological involvement of heart, esophagus, colon, or a combination, and are grouped into three major forms: cardiac, digestive, and cardio-digestive (Marin Neto JA *et al*., 1999).

The digestive form is seen mainly in regions south of the Amazon basin (Argentina, Brazil, Chile, and Bolivia). This geographical distribution is most likely due to differences in parasite strains (Miles MA *et al*., 2003). Gastrointestinal dysfunction (megaesophagus and/or megacolon) arises in 10-15% of chronically infected patients. The megaoesophagus can lead to dysphagia (difficulty in swallowing) and odynophagia (pain during swallowing), together with epigastric pain, regurgitation, and malnutrition in severe cases. There is also an increased risk of cancer of the esophagus in CD patients with megaesophagus. Megacolon, which commonly affects the sigmoid colon and rectum, leads to severe constipation, abdominal distension and pain, and occasionally results in a large bowel obstruction due to fecaloma or sigmoid volvulus (Brandalise NA *et al*., 1985).

The cardiac form is the most frequent and serious manifestation of chronic CD. It develops in 20-30% of individuals and usually leads to abnormalities of the conduction system, tachyarrhythmias and bradyarrhythmias, apical aneurysms, thromboembolism, heart failure, and sudden death (Marin Neto JA *et al*., 1999; Rassi A Jr *et al*., 2000). Common ECG abnormalities include right bundle branch block, left anterior fascicular block, abnormal Q waves, premature (ectopic) ventricular beats, and low voltage of QRS complex. Episodes of non-sustained ventricular tachycardia are observed in 40% of patients with mild abnormalities of wall motion, and in almost all patients with HF, which is more common than in other cardiomyopathies (Rassi A Jr *et al*., 1995).

HF is often a late manifestation of CD. It is mostly biventricular with a predominance of right-sided failure (peripheral edema, ascites, and hepatomegaly are more prominent than pulmonary congestion) at advanced stages. Isolated left-sided HF can be present in the early stages of cardiac decompensation. HF due to CD is associated with higher mortality than is HF from other causes (Freitas HF *et al*., 2005).

Systemic and pulmonary embolisms arising from mural thrombi in the cardiac chambers are quite common, especially in the brain, lungs, and limbs (Oliveira JSM *et al*., 1983). As a result, CD is an independent risk factor for stroke in endemic countries (Carod-Artal FJ *et al*., 2005).

Sudden death is the major cause of death in patients with cardiomyopathy due to CD. It accounts for almost two-thirds of all deaths, followed by refractory HF (25-30%), and thromboembolism (10-15%) (Rassi A Jr. *et al*., 2001). Sudden cardiac death can also occur in patients who were previously asymptomatic. It is commonly associated with ventricular tachycardia and fibrillation or, less likely, with sinus node dysfunction or complete atrioventricular block. The leading causes of death vary depending on the stage of the disease, with sudden death being predominant at early stage, and death from HF being more common at advanced stages.

There might be an exacerbation of chronic infection in patients with AIDS or those receiving immunosuppressive therapy, which can lead to increases in parasitemia and replication of intracellular parasite. Fever, skin lesions, myocarditis, and panniculitis are common in recipients of solid organs or bone marrow transplants (Altclas J *et al*., 2005; Fiorelli AI *et al*., 2005), whereas meningoencephalitis and myocarditis are more commonly seen in patients with AIDS (Vaidian AK *et al*., 2004; Sartori AM *et al*., 2007).

1.1.3.4.   Epidemiology of Chagas' disease

CD is endemic throughout most of South and Central America, including some southern regions of United States especially the state of Texas (Hotez PJ, 2014). Originally it was confined to the poor, rural areas of Latin America, in which vector-borne transmission to man occurs (Moncayo A, 2003).

In countrywide surveys in 1980s, nearly 17.4 million people were estimated to be infected, and 100 million (i.e. 25% of all inhabitants of Latin America) were at risk of infection in 18 endemic countries in 1980-85 (WHO 2002). According to the Pan American Health Organization, 20% of population of Latin America were at risk (109 million individuals) and approximately 7.7 million individuals were infected in 2005

(Aguilar VHM *et al*., 1999). In a recent Chagas' disease factsheet by WHO, it is estimated that 10 million people are infected worldwide, mostly in Latin America where CD is endemic, and more than 25 million people are at risk of the disease. It is estimated that in 2008, CD killed more than 10,000 people (Chagas disease [American trypanosomiasis] fact sheet [revised in June 2010]).

In the past few decades, improved programs for vector control and compulsory blood bank screening have significantly reduced new cases of infection and decreased the burden of CD in Latin America. The incidence of new cases of CD dropped from 700,000 per year in 1990 to 41200 per year in 2006. Similarly, number of deaths from CD fell from 50,000 per year in 1990 to 12,500 per year in 2006 (Moncayo A and Silveira AC, 2009). However, the recent influx of immigrants from countries endemic for disease has meant that CD is becoming a major health concern in USA and Canada and in many parts of Europe and the western Pacific, where an increasing number of infected individuals have been identified (Schmunis GA, 2007; Guerri-Guttenberg RA *et al*., 2008). The most common destination for migrants from Latin America is the USA, where an estimated 300,167 individuals (mainly from Mexico) are infected with *T.cruzi* (Bern C and Montgomery SP, 2009). Spain has the second highest number of infected immigrants, with an estimated 47,738 - 67,423 individuals, most of them originating from Argentina, Ecuador, Bolivia and Peru (Gascon J *et al*., 2010). Other countries like France, Canada, Japan, and Australia have also reported cases of CD and are at increasing risk due to flow of migrants.

1.1.3.5.   Diagnosis of Chagas' disease

For diagnosis of an acute infection, microscopic examination of peripheral blood is done for detection of trypomastigotes (Gomes YM *et al*., 2009). Micro-hematocrit is the preferred method for diagnosis of congenital infections as it has a high sensitivity and only a small amount of blood is required. Microscopic examination of cord or peripheral blood by this method is highly recommended in neonates (Bittencourt AL, 1976; Freilij H and Altcheh J, 1995). In case results are repeatedly negative or test is not done early in life, then the infant should be tested for anti-*T.cruzi* IgG antibodies at 6-9 months of age when maternal antibodies are no longer present in infant's serum. In specialized centers, PCR (polymerase chain reaction) techniques based upon amplification of *T.cruzi* DNA detection, can be used for early detection of congenital infections. This method tends to

have a higher sensitivity than microscopic examination (Mora MC *et al*., 2005; Russomando G *et al*., 2005; Diez CN *et al*., 2008).

In cases of chronic infection, as the parasitemia is very low, direct microscopic examination of patient's blood is no longer a reliable method. Instead the presence of IgG antibodies against *T.cruzi* antigens is detected by at least two different serological methods (usually enzyme linked immunosorbent assay, indirect immunofluorescence, or indirect haemagglutination) to confirm diagnosis (Gomes YM *et al*., 2009). PCR is not used in routine diagnosis because of the need for specific laboratory facilities, poor standardization, potential DNA cross-contamination, and variable results across laboratories and countries. However, due to its high sensitivity compared with other parasitological methods, PCR can be potentially used for confirmation of diagnosis in cases of inconclusive serology, and as an additional method to monitor treatment (Britto CC, 2009). It can be used to identify treatment failure from positive detection of *T.cruzi* DNA, but not for treatment success, since even repeated negative PCR results do not necessarily indicate complete cure.

1.1.3.6.   Treatment of Chagas' disease

1.1.3.6.1. Anti-trypanosomal treatment

Anti-trypanosomal treatment is strongly recommended for all cases of acute, congenital, and reactivated infection, for all children with infection, and for patients up to 18 years of age with chronic disease. Drug treatment should be offered to adults aged 19-50 years without advanced CD, and is optional for those older than 50 years because benefit has not been proven in this group of population (Bern C *et al*., 2007). Anti-trypanosomal treatment is contraindicated during pregnancy and in patients with severe hepatic or renal insufficiency, and generally is not offered to patients with advanced CD or megaoesophagus with profound impairment of swallowing.

Benznidazole and nifurtimox are the drugs of choice, having proven efficacy against CD (de Andrade AL *et al*., 1996; Sosa Estani S *et al*., 1998; Viotti R *et al*., 2006; Fabbro DL *et al*., 2007). Benznidazole has the best safety and efficacy profile (Viotti R *et al*., 2009), and is therefore usually used as first-line treatment.

1.1.3.6.2. Treatment of cardiac symptoms

Amiodarone can improve survival in patients with cardiomyopathy due to CD who are at high risk of death from malignant arrhythmias (Scanavacca MI *et al*., 1990; Rassi A Jr *et al*., 1995; Rassi A Jr *et al*., 2001). It is therefore commonly recommended as the treatment

of choice for all patients with sustained ventricular tachycardia and for those with non-sustained ventricular tachycardia and myocardial dysfunction (Scanavacca MI *et al*., 2002).

Angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) are the mainstay of treatment for CD patients with HF. Diuretics are also frequently used due to predominance of systemic congestive symptoms over signs of pulmonary congestion. However, ß blockers are not used as frequently as they are in HF from other causes. This is due to the reason that CD patients with cardiomyopathy also have profound conduction abnormalities and tend to have symptomatic bradycardia that can be exacerbated with the use of ß blockers (Quiros FR *et al*., 2006).

Cardiac resynchronization is another form of treatment for HF and is mostly administered to patients with left bundle branch block. Cardiac transplantation is an effective alternative for patients with end stage HF (Bocchi EA and Fiorelli A, 2001). Due to the high occurrence of thromboembolic conditions, oral anticoagulants are recommended for patients with atrial fibrillation, previous embolism, and apical aneurysms with recent thrombi.

1.1.3.6.3. Treatment of gastrointestinal symptoms

Treatment of mega-esophagus is focused on palliation of symptoms by aiding the transit of food and liquids through the achalasic lower esophageal sphincter. Surgical techniques like laparoscopic Heller's myotomy and fundoplication are used for treatment of non-advanced mega-esophagus. In advanced cases, different techniques of esophageal resection are used (Herbella FA *et al*., 2008).

The early stage of colonic dysfunction can be treated with a fiber-rich diet and abundant intake of fluids, together with laxatives and occasional enema. Fecal impaction can occur as the disease progresses requiring manual emptying under general anesthesia. Patients with megacolon who do not respond to conservative treatment and those with frequent fecaloma or sigmoid volvulus, need to undergo surgical organ resection (Garcia RL *et al*., 2008).

1.1.3.7. Economic burden of Chagas' disease

Regarded by WHO as one of the thirteen most neglected tropical diseases in the world (Hotez PJ *et al*., 2007), CD continues to be a major source of financial, social and health burden to the affected individuals and countries (Mathers CD *et al*., 2007). The economic impact of the disease especially during chronic phase is very high as it represents the most

common cause of cardiac lesions in the young, economically productive adults in endemic countries of Latin America (Moncayo A and Silveira AC, 2009). The early mortality and severe disability associated with CD results in huge financial losses every year in these developing countries. In Latin America, CD holds fourth place amongst infectious and parasitic diseases with the largest burden of the disease. It is associated with 0.7 million disability-adjusted life years (DALYs), just behind diarrheal diseases, HIV, and tuberculosis (Hotez PJ *et al.*, 2006).

Poor housing conditions, lack of screening at blood banks, inadequate prenatal and health care facilities have resulted in the persistence of CD in many areas of Latin America that are still developing. It has put a massive burden on poor families in rural areas, as the financial, social and health consequences of the disease only contribute towards maintaining the cycle of poverty. Furthermore, a huge load is also put on the health care system due to hospitalizations and medical and surgical treatments, especially as a consequence of chronic manifestations of disease like cardiomyopathy, gastrointestinal dysfunction, and meningo-encephalitis. The annual financial loss due to early morbidity and mortality from CD in Latin America is estimated to be as high as 18 billion dollars (Franco-Paredes C *et al.*, 2007).

1.1.3.8.   Worldwide importance of Chagas' disease

Due to the recent advancements in communication and technology, it has become much easier to travel from one part of the world to another. However, globalization phenomenon has also enabled the spread of infectious diseases across countries and continents to regions where it previously did not exist, or had been eradicated only to see it re-emerge again. CD was originally confined to the rural areas, where poor housing conditions and inadequate health care facilities favour its existence. However, with the rising urbanization trend, where people are migrating to big cities for better opportunities, CD is now no longer limited to the endemic, rural areas of Latin America. A large number of people are also migrating from CD endemic countries to more developed countries, especially in North America (USA and Canada) and Europe.

The spread of CD to non-endemic regions has largely been facilitated by the fact that CD is transmissible by blood transfusion and organ transplantation, and there are no specific tests at blood banks or hospitals to screen it. As a result, there are already several registered cases of infection via blood transfusion and organ transplantation in United

States and Europe (Centers for Disease Control and Prevention [CDC], 2006; Young C *et al.*, 2007; Florez-Chavez M *et al.*, 2008).

Vertical transmission is another way of spreading the disease as children born with *T.cruzi* infection mostly remain undiagnosed, transmitting the disease later on in life via blood transfusion or organ donation, or in the case of women, giving birth to infected newborns, thus perpetuating the cycle of congenital transmission (Muñoz J *et al.*, 2007).

1.2.   Biomarkers in cardiac diseases

1.2.1. Introduction to biomarkers

The accurate diagnosis and prevention of cardiac diseases is an important public health goal. Although clinical characteristics such as age and gender are well established risk factors for cardiac diseases, such features are not sufficient to identify all patients at risk. Cardiac biomarkers are one such tool to better identify high-risk individuals, to diagnose disease conditions promptly and accurately, and to effectively prognosticate and treat patients with disease. However, most current biomarkers have only modest predictive value, and there is a need to identify additional biomarkers from new biological pathways. A biomarker is actually defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention (Biomarkers Definitions Working Group, 2001). It may be measured on a bio-sample (as a blood, urine, or tissue test), it may be a recording obtained from a person (blood pressure, ECG, or Holter), or it may be an imaging test (echocardiogram or CT scan). Accordingly, biomarkers can be classified as antecedent biomarkers (identifying the risk of developing an illness), screening biomarkers (screening for subclinical disease), diagnostic biomarkers (recognizing overt disease), staging biomarkers (categorizing disease severity),or prognostic biomarkers (predicting future disease course, including recurrence and response to therapy, and monitoring efficacy of therapy).

The overall expectation of a cardiac disease biomarker is to enhance the ability of the clinician to optimally manage the patient. A new biomarker will be of clinical value only if it is accurate and is reproducibly obtained in a standardized fashion. It should be acceptable to the patient and easy to interpret by clinicians. In addition, it should have high sensitivity and high specificity for the outcome it is expected to identify (Fox N and Growdon JH, 2004).

1.2.2. Role of biomarkers in heart failure

HF appears to result not only from cardiac overload or injury but also from a complex interaction among various genetic, neurohormonal, inflammatory, and biochemical factors that act on the cardiac myocytes and the interstitium. An increasing number of enzymes, hormones, biologic substances, and other markers of cardiac stress and injury are gaining clinical importance as biomarkers. These biomarkers provide important information regarding the identification of individuals at risk of HF, diagnosis of HF, risk stratification and in monitoring therapy, as well as about the pathogenesis of HF. Many biomarkers may be risk factors themselves and therefore potential targets of therapy. As inflammation is important in the pathogenesis and progression of many forms of HF, various inflammatory biomarkers have been identified that have proved useful in HF. These include C-reactive protein (CRP), TNF alpha, and interleukins1, 6, and 10 (Anker SD and von Haehling S, 2004). Neurohormonal disturbances also play a role in the pathogenesis of HF, including the activation of the renin-angiotensin-aldosterone system, and subsequent stimulation of sympathetic nervous system, leading to abnormally elevated levels of norepinephrine in plasma (Cohn JN *et al*., 1984; Swedberg K *et al*., 1990). Brain natriuretic peptide (BNP) is an important indicator of myocyte stress. It is formed by the myocytes during hemodynamic stress such as when the ventricles are dilated, hypertrophic, or subject to increased wall tension. BNP causes arterial vasodilation, diuresis, and natriuresis, and reduces the activities of the renin-angiotensin-aldosterone system and the sympathetic nervous system (Moreira Mda C *et al*., 2008). Thus, BNP acts to oppose the physiologic abnormalities in HF. It serves as a useful biomarker in the diagnosis and risk stratification of patients with chronic HF. Also, BNP together with atrial natriuretic peptide (ANP) are effective in screening of asymptomatic patients at risk of developing HF, such as the elderly and those with diabetes and hypertension. N-terminal pro-BNP, due to its longer half-life, is more accurate as an indicator for ventricular stress and for prognosis (Masson S *et al*., 2006). However, BNP cannot be used as a biomarker for differentiating between CD and DCM due to other causes. Chagas' serology and echocardiography must be performed additionally to differentiate between CD and DCM.

1.2.3. Role of biomarkers in Chagas' disease

The clinical manifestations of CD are variable, but most of the individuals infected with *T.cruzi* remain asymptomatic for decades. Therefore, transmission of CD via blood

transfusion and organ transplantation still occurs in countries where CD is endemic and in those where it is not. Vertical transmission is also a possibility as CD can pass unsuspected from mother to child for several generations. All the current diagnostic and screening tests have limitations. Therefore, new approaches are needed for blood bank screening as well as for improved diagnosis and prognosis. There is no gold standard assay for CD, but tests currently in use include enzyme- linked immunosorbent assay (ELISA), PCR, indirect immunofluorescence assay (IFA), and xenodiagnosis. Although serologic screening tests have greatly reduced the risk of transmission of CD, these methods are still imperfect and better tests are needed to protect transfusion and transplant recipients worldwide. In recent times, there have been various studies to identify useful biomarkers for early screening and diagnosis of CD. A new ELISA technique, the Ortho *T.cruzi* ELISA test system, was approved by the FDA for screening of blood donors and was licensed in December 2006 (Gorlin J *et al*., 2008). Recently, specific diagnostic serum biomarkers for CD in asymptomatic patients have been identified, using mass spectrometric profiling. These include macrophage inflammatory protein-1alpha (MIP-1alpha), C3a anaphylatoxin, and unusually truncated forms of fibronectin, apolipoprotein A1 (ApoA1), and C3 (Ndao M *et al*., 2010). However, it still remains to be seen how effective these biomarkers can be in accurate screening and diagnosis of CD.

1.2.4. Potential role of biomarkers in cardiomyopathy due to Chagas' disease

Cardiac involvement is the most common manifestation of chronic CD, and represents the major cause of mortality. However, its diagnosis is still based on nonspecific criteria with poor sensitivity. Early identification of patients with cardiomyopathy due to CD is desirable, as early therapy may improve prognosis. Plasma BNP concentration and plasma angiotensin converting enzyme-2 (ACE-2) activity are important biomarkers for HF, and can potentially be used for diagnostic and prognostic purposes in patients with CD (Moreira Mda C *et al*., 2008; Wang Y *et al*., 2010). Infact, BNP and ACE-2 have additive predictive value, and may be used in combination for prediction of HF in patients with CD. Similarly, inflammatory biomarkers can play a vital role in early diagnosis, as inflammation of heart is a major hallmark of cardiomyopathy due to CD, and may be present even in the absence of HF. Plasma levels of TNF alpha, IL-6, and endothelin 1, are elevated in patients with CD (Garcia-Alvarez A *et al*., 2010). Thus, they may be used as biomarkers for early diagnosis and treatment of patients with cardiac involvement due to CD.

1.3.   Cytokines – a brief overview

Cytokines are small proteins that play an important role in intercellular signaling. They are produced by various cell types in the body including lymphocytes (lymphokines) and monocytes (monokines). Cytokines which are produced by leukocytes and in turn act on other leukocytes are called interleukins. Cytokines can exert their effect on cells that produce them (autocrine effect), on neighbouring cells (paracrine effect), and also on remote cells in the body (endocrine effect) (Zhang JM and An J, 2007).

Cytokines regulate host responses to infection, immune responses, inflammation, and trauma. Some cytokines are involved in the up-regulation of inflammatory reactions. These are known as pro-inflammatory cytokines and are mainly produced by activated macrophages. TNF alpha and IL-1 are some examples of pro-inflammatory cytokines (Dinarello CA *et al*., 2000). Anti-inflammatory cytokines, on the other hand, are immune-regulatory molecules that control the pro-inflammatory cytokine response. Such cytokines work together with specific cytokine inhibitors and soluble cytokine receptors to regulate the immune response in human body. Some of the anti-inflammatory cytokines present in the body include IL-1 receptor antagonist, IL-4, IL-6, and IL-10 (Opal SM and DePalo VA, 2000).

Chemokines are a family of small cytokines secreted by cells. Some of these chemokines actively participate in the inflammatory response to an infection, attracting immune cells to the site of inflammation (Mélik-Parsadaniantz S and Rostène W, 2008).

There are certain cytokines also present in the human body that exert diverse immune effects. Leukemia inhibitory factor (LIF), for example, is one such cytokine. In some conditions, LIF can act as a pro-inflammatory cytokine, inducing the production of other pro-inflammatory cytokines and monocyte chemo-attractants, while in other conditions it can suppress inflammation and act as an anti-inflammatory cytokine (Alexander HR *et al*., 1994).

1.4.   Aims of Study

CD is one of the most important neglected tropical diseases in the world. It continues to be a source of great financial and social burden in Latin America and now is a major concern for countries outside Latin America due to rising risk of non-vectorial transmission by blood transfusion and organ transplantation. Thus, there is a growing need for development of improved serological and molecular tests for effective screening in blood banks and hospitals, and for accurate diagnosis, that would be essential to initiate

appropriate therapy. Moreover, reliable prognostic tools are required for developing risk stratification models for mortality in CD patients that would help in selecting the best possible treatment regimen.

Keeping in view the inflammatory nature of CD, this study investigated the possible role of serum cytokines as biomarkers for prediction and prognosis of CD. The serum levels of 21 different inflammatory cytokines were measured in a group of patients with CD and then compared with those measured in patients with DCM from other causes, and with control subjects. The inflammatory cytokines that were studied included:interleukin-1alpha (IL-1alpha), interleukin-16 (IL-16), interleukin-18 (IL-18), macrophage-colony stimulating factor (M-CSF), macrophage migration inhibitory factor (MIF), stem cell factor (SCF), stem cell growth factor beta (SCGF beta), tumour necrosis factor beta (TNF beta), TNF-related apoptosis inducing ligand (TRAIL), interleukin-12p40 (IL-12p40), interleukin-2 receptor alpha (IL-2R alpha), interleukin-3 (IL-3), hepatocyte growth factor (HGF), interferon-alpha2 (IFN-alpha2), beta-nerve growth factor (beta-NGF), leukemia inhibitory factor (LIF), cutaneous T-cell attracting chemokine (CTACK), growth related oncogene alpha (GRO alpha), monocyte chemo-attractant protein-3 (MCP-3), monokine induced by interferon gamma (MIG), and stromal derived factor-1alpha (SDF-1alpha).

2. Materials and Methods

2.1. Instruments

Bio-Plex® or Luminex system	Bio-Rad Laboratories, Hercules, 94547, CA, USA.

Bio-Plex validation kit

Bio-Plex calibration kit

Bio-Plex Pro II wash station

Bio-Plex Pro flat bottom

plates (40, 1×96-well plates)

Microtiter plate shaker	BioTech, Ritterstieg,Germany.
Vortexer	VWR, Leuven, USA.

Reagent reservoirs, 25 ml

(for captured beads and

detection antibodies)

Reagent reservoir, 50 ml	Greiner Bio-One, Frickenhausen,
(for reagent and buffers)	Germany.

Pall Life Science Acrodisc:

25 mm PF syringe filter

(0.8/0.2 μm Supor membrane)

Ultrasonographic system	Sequoia C256, Acuson, Acuson Inc, Mountain View, CA, USA.

2.2. Materials

Bio-Plex Pro assay kit:	Bio-Rad Laboratories, Hercules, CA, USA

Coupled magnetic beads (10×)

Detection Antibodies (10×)

Universal standard diluent

Universal sample diluents

Assay buffer

Wash buffer

Detection antibody diluent

Streptavidin-PE (100×)

Filter plate (96-well)

Sealing tape

Tube holder

Sterile distilled water

Aluminium foil

Absorbent paper towels

Micro-centrifuge tubes (1.5 or 2 ml)

2.3. Methods

2.3.1. Patients

The study was approved by the institutional review committee, and all patients gave written consent. The study population consisted of a prospective cohort of 142 subjects from the Heart Failure Center of the Felicio-Rocho Hospital, Brazil, enrolled between July 2001 and January 2005. Ninety- four (94) consecutively recruited patients with atleast two positive serologies for CD (Chagas group [CD]) and forty-eight (48) patients with negative serology for CD (Idiopathic DCM group) were studied and compared with twenty-five (25) adjusted gender- and age-matched healthy subjects.

All clinical data was obtained by the same investigator and included the medical history, physical examination, and resting electrocardiogram. M-mode, two-dimensional, and Doppler echocardiographic measurements were performed with an ultrasonographic system (Sequoia C256, Acuson, Acuson Inc, Mountain View, CA) according to the recommendations of American Society of Echocardiography. Left ventricular systolic dysfunction was defined by a left ventricular ejection fraction (LVEF) of $< 50\%$ as assessed by transthoracic echocardiography (Teichholz method).

Other structural cardiac diseases and co-morbidities were excluded by medical history, physical examination, electrocardiogram, M-mode, two-dimensional, and Doppler echocardiography, or coronary arteriography: valvular heart disease, coronary artery disease,

congenital disease, acute myocarditis, hypertensive disease, renal failure (plasma creatinine > 0.2 mmol/L), chronic pulmonary disease, hepatic cirrhosis, active infections, and endocrine disease. Patients with ventricular dysfunction received standard pharmacologic therapy according to the New York heart association (NYHA) functional class and were clinically stable for at least 30 days.

Patients with advanced refractory HF were considered as heart transplant candidates and listed for the procedure in the absence of any contraindication. The control group was composed of healthy subjects attending the hospital clinics for a physical check-up. They had no cardiac symptoms or history, no co-morbidities, and took no medication.

The study patients were prospectively defined and subdivided into 5 groups: Group 1 (n = 46), CD without ventricular systolic dysfunction (LVEF > 50%); Group 2 (n = 25), CD with ventricular systolic dysfunction (LVEF < 50%) in NYHA classes I-II; Group 3 (n = 23), CD with ventricular systolic dysfunction (LVEF < 50%) in NYHA classes III-IV; Group 4 (n= 22), DCM with ventricular systolic dysfunction (LVEF < 50%) in NYHA classes I-II; and Group 5 (n = 26), DCM with ventricular systolic dysfunction (LVEF < 50%) in NYHA classes III-IV.

Patients were followed for incidences of cardiac death or heart transplant from the time the blood sample was obtained for measurement and analysis of cytokines until end points were reached or until the follow-up closing date in January 2006. The end point was defined as either heart transplantation or death. Long-term follow-up of each patient was conducted by medical visit or telephone interview every three months. For those hospitalized during follow-up, the hospital records were reviewed. For all subjects who died, the nearest relative was contacted.

2.3.2. Plasma sampling

Ten milliliters of blood samples were taken from the antecubital vein of subjects and transferred into tubes containing ethylenediamine tetra-acetic acid (EDTA). Immediately after sampling, plasma was separated by centrifugation at 4000g for 10 minutes, and frozen at -80°C for further analysis. To prepare each sample, 35µL of EDTA was taken and diluted with a sample diluent. Samples were kept on ice till they were ready for use.

2.3.3. Cytokines measurement

Plasma concentrations of twenty-one (21) different human cytokines were measured using the Bio-Plex Pro^{TM} system that combined the principle of a sandwich immunoassay with the Luminex fluorescent-bead-based technology (Bio-Rad Laboratories) (Fu Q *et al*., 2010). An antibody directed against the desired cytokine, chemokine, or growth factor target is covalently coupled to internally dyed beads. The coupled beads are allowed to react with the sample containing the target bio-molecules. After a series of washes to remove the unbound protein, a bio-tinylated detection antibody which is specific to an epitope different from that of the capture antibody is added to the reaction. This results in the formation of a sandwich of antibodies around the cytokine, chemokine, or growth factor target. A streptavidin-phycoerythrin (streptavidin-PE) reporter complex is then added to bind to the biotinylated detection antibodies on the bead surface. Data from the reaction are acquired using the Bio-plex system, and analyzed by Bio-Plex Manager^{TM} software.

The Bio-Plex$^{®}$ system is built around three core components. The first is a system of 100 unique fluorescently dyed beads (xMAP technology) that permit the simultaneous detection of up to 100 different types of molecules in a single well of a 96-well micro-plate. The second component is a flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads. The third is a high-speed digital signal processor that efficiently manages the fluorescent data.

2.3.4. Data acquisition and analysis

Data from the reaction are acquired using the Bio- Plex$^{®}$ system (or Luminex system), a dual-laser, flow-based micro-plate reader system. The contents of the plate are drawn up into the reader. The lasers and associated optics detect the internal fluorescence of the individual dyed beads as well as the fluorescent reporter signal on the bead surface. This identifies each assay and reports the level of target protein in the sample. Intensity of fluorescence detected on the beads will indicate the relative quantity of the target bio-molecules in the tested samples. A high-speed digital processor then manages the data output, which is further analyzed and presented as fluorescence intensity (FI) and target concentration on Bio-Plex Manager^{TM} software. Results were expressed in picograms per milliliter (pg/mL).

2.3.5. Missing values analysis

Cases with missing values represent an important challenge, as discarding these cases from the analysis can significantly reduce the amount of data. When there are only few values that are missing then the standard method of list-wise deletion is relatively "safe". However, this deletion may decrease the precision of calculated statistics as there is less information than originally planned. Therefore, the method of imputing can help by providing some surrogates of the missing values. This procedure provides multiple versions of the dataset, each containing its own set of imputed values (5 imputing datasets were used in this analysis). For statistical analysis, the parameter estimates for all of the imputed datasets are pooled, providing more accurate statistics. Missing values analysis was performed using IBM SPSS version 18.

(Dr Niels Wessel [Charite, Berlin, Germany] supported and assisted in the statistical analysis.)

2.3.5.1. Chagas' disease

Dataset contained 111 cases. 17 cases did not have any of the 21 parameters. They were all excluded from further analysis. Twelve values were imputed for 94 cases:

IL-1alpha, IL-18, M-CSF, TNF beta, IL-12p40, IL-3, IFN-alpha2, beta-NGF, LIF, GRO alpha, MCP-3, and SDF-1alpha were imputed.

Other nine values were not necessary to impute as they were available for all cases.

2.3.5.2. Dilated cardiomyopathy

Dataset contained 62 cases. 14 cases did not have any of the 21 parameters. They were excluded from further calculations. Five values were imputed for 48 cases:

IL-1alpha, TNF beta, LIF, GRO alpha, and MCP-3 were imputed.

Other sixteen values were fully available for all 48 patients.

2.3.6. Statistical analysis

The plasma concentrations of all 21 cytokines were expressed as mean ± standard error of the mean (SEM). The associations between concentrations of various cytokines and clinical variables were tested using Mann-Whitney U test. As the data was normally distributed, Pearson's correlation coefficient (r) was used to analyze the correlation between the

investigated cytokines in CD and DCM patient groups and various echocardiographic parameters – they were tested against the null hypothesis that the correlation coefficient is 0. A p value < 0.05 was defined as significant. Kaplan-Meier curves were also drawn to compare the survival or necessity for heart transplantation of patients with CD and DCM depending on different concentrations of cytokines.

3. Results

3.1. Patients

Baseline characteristics of patients and control subjects according to the NYHA functional class, ECG, echocardiographic parameters, and medications are given in **table 1**. The mean duration of follow-up was 36.9 months (range: 13-54 months). At the end of study, the survival status of all patients was known; 30 patients had died and 12 patients had received a heart transplant. Of these, 18 patients with CD and 12 patients with DCM had died. While 6 patients each from CD and DCM groups had received a heart transplant.

Systolic blood pressure was significantly altered in patients in the CD group and in patients in the DCM group with advancing HF in comparison with the control group or patients with CD without ventricular systolic dysfunction. **Table 1** also illustrates increased impairment of cardiac function in patients with CD and in patients with DCM with increasing NYHA class.

All patients with ventricular dysfunction received maximal medical therapy according to their functional classes and the treatment guideline recommendations, as shown in **table 1**. Almost all patients with ventricular dysfunction were given ACEIs or ARBs; 22 patients with DCM were taking carvedilol, but only 3 patients with CD were receiving beta blocker therapy. This lack in beta blocker treatment in patients with CD, although recommended by all guidelines for the treatment of HF, resulted from missing experience with beta blockers in CD because all the major trials on HF exclude patients with CD. Moreover, this disease is very often characterized by bradyarrhythmias and advanced atrioventricular block, which makes it difficult to use beta blockers (Braga JC *et al.*, 2006; Wang Y *et al.*, 2010).

3.2. Data analysis

Plasma concentrations of 21 different cytokines were measured in control subjects, in CD patients, and in DCM patients **(Table 2)**. The CD patients were divided into asymptomatic (0), NYHA classes I-II, and NYHA classes III-IV. While DCM patients were divided into NYHA classes I-II and NYHA classes III-IV. Cytokines were also subdivided into 4 groups: pro-inflammatory cytokines, anti-inflammatory cytokines, cytokines with diverse immune effects, and chemokines.

Groups	Control	Chagas' disease			DCM	
		0	NYHA I-II	NYHA III-IV	NYHA I-II	NYHA III-IV
	(n = 25)	(n = 46)	(n = 25)	(n = 23)	(n = 22)	(n = 26)
Age (years)	53.9 ± 2.9	52.0 ± 1.5	50.6 ± 2.3	48.9 ± 1.9	49.8 ± 3.6	47.2 ± 2.4
Sex						
Male	12	11	10	13	14	16
Female	13	35	15	10	8	10
SBP (mmHg)	121.0 ± 2.1	125.7 ± 2.1	110.3 ± 2.2###	98.7 ± 2.9***/###	112.8 ± 2.8#	95.9 ± 4.6***/###
DBP (mmHg)	76.4 ± 0.98	78.9 ± 1.2	73.3 ± 1.3	70.3 ± 1.8###	73.9 ± 1.5	70.8 ± 1.8###
HR (beats/min)	73.6 ± 1.2	69.5 ± 1.3	69.8 ± 2.1	75.0 ± 2.5	72.9 ± 2.0	83.2 ± 2.2**/###
ECG						
Normal/Abnormal	25/0	13/33	0/25	0/23	0/22	0/26
Pacemaker	0	4	6	3	1	1
ECHO						
LVEF (%)		67.5 ± 1.1	37.8 ± 2.0###	24.5 ± 1.5###	37.2 ± 2.2###	23.4 ± 1.3###
LVEF>50%/<50%		46/0	2/23	0/23	0/22	0/26
LVDD (mm)		51.9 ± 1.0	62.7 ± 1.6###	70.9 ± 1.3###	64.7 ± 2.1###	74.9 ± 2.0###
LVSD (mm)		32.6 ± 0.90	50.6 ± 1.8###	62.1 ± 1.3###	52.8 ± 2.1###	66.6 ± 2.1###
LVEDV (ml)		131.5 ± 6.8	204.1 ± 12.0#	303.4 ± 20.2###	217.7 ± 16.8#	358.4 ± 36.3###
LVESV (ml)		41.8 ± 3.0	109.8 ± 10.7##	209.5 ± 10.7###	137.5 ± 13.5###	256.9 ± 28.8###
Pharmacotherapy						
No medication	25	31	1	-	-	-
ACEI	-	3	22	21	20	15
ß-blocker	-	1	2	-	14	8
ARB	-	-	1	1	2	8
Amiodarone	-	4	5	6	3	2
Diuretic	-	3	17	21	19	25
Digoxin	-	2	11	19	12	22
Spironolactone	-	-	4	14	13	19
Anticoagulant	-	3	2	6	2	8

Table 1. Patient characteristics. Data given as mean ± S.E.M. ****** $p < 0.01$, ******* $p < 0.001$ *vs.* Control; **#** $p < 0.05$, **##** $p < 0.01$, **###** $p < 0.001$ *vs.* Chagas group 0. DBP = Diastolic blood pressure, HR = Heart rate, LVDD = Left ventricular diastolic diameter, LVEDV = Left ventricular end-diastolic volume, LVESV = Left ventricular end-systolic volume, LVSD = Left ventricular systolic diameter, SBP = Systolic blood pressure.

Cytokines	Control		CD 0		CD I-II		CD III-IV		DCM I-II		DCM III-IV	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL-1alpha	14.63	3.847	15.38	2.889	16.19	4.152	12.71	3.570	13.78	3.815	13.79	5.118
IL-16	319.6	28.94	309.7	19.14	322.5	19.16	344.6	30.24	374.9	30.76	354.8	22.25
IL-18	139.1	22.19	146.2	21.29	134.4	20.60	171.2	28.60	182.9	31.81	157.5	30.18
M-CSF	28.02	5.254	24.71	4.285	28.01	4.860	35.55	6.818	27.22	6.688	26.97	5.445
MIF	162.9	19.17	206.1	24.25	189.3	26.30	324.5	64.53	171.0	21.61	196.9	20.72
SCF	195.0	18.49	196.0	16.62	201.5	20.89	223.1	26.91	219.9	26.05	228.4	23.00
SCGF beta	24,130	4,856	22,943	2,638	26,103	5,226	46,231	9,495	28,944	6,168	45,903	7,705
TNF beta	17.04	3.293	14.88	2.330	15.35	2.873	12.90	2.438	18.48	3.788	14.62	4.146
TRAIL	148.0	16.86	152.7	11.28	182.9	24.80	192.3	26.14	170.0	21.80	186.1	28.47
IL-12p40	130.6	19.74	171.9	17.18	178.5	27.67	260.3	50.42	172.0	26.48	246.0	41.79
IL-2R alpha	100.9	10.32	102.7	7.300	104.6	12.28	123.0	15.98	95.63	7.602	125.1	12.53
IL-3	73.29	8.889	82.60	7.425	79.53	8.999	91.00	10.34	84.69	9.154	98.01	9.559
HGF	240.2	27.36	242.5	16.88	284.0	36.50	649.0	91.38	321.4	30.76	550.5	54.94
IFN-alpha2	57.79	4.081	57.73	3.088	58.36	3.912	63.97	5.431	61.84	3.949	69.96	3.476
beta-NGF	18.30	4.129	16.28	2.615	19.91	4.375	22.51	4.439	22.44	5.592	26.23	5.866
LIF	76.14	4.100	51.46	7.563	53.96	9.145	44.19	9.091	54.69	11.09	56.71	10.79
CTACK	1,436	77.27	1,317	70.09	1,519	121.4	1,798	220.3	1,504	100.5	1,480	100.5
GRO alpha	45.48	8.952	42.44	6.218	48.10	6.131	56.01	7.865	39.94	4.866	42.47	4.872
MCP-3	19.74	3.743	17.26	1.462	15.96	2.099	24.72	4.895	15.75	2.846	23.65	3.198
MIG	808.4	147.4	1224	162.7	1,695	324.3	2,329	411.5	832.8	95.29	1,307	211.3
SDF-1alpha	138.6	21.32	173.7	18.30	178.8	26.58	214.2	35.44	164.7	24.17	266.5	29.98

Table 2. Plasma concentrations of cytokines in control, CD, and DCM groups expressed as mean ± standard error of the mean (SEM). All values are expressed in pg/mL.

3.2.1. Pro-inflammatory cytokines

Pro-inflammatory cytokines in this study included: IL-1alpha (Piper SC *et al*., 2013), IL-16 (Reikeras O *et al*., 2014), IL-18 (Piper SC *et al*., 2013), M-CSF (Vincent VA *et al*., 2002), MIF (Al-Abed Y *et al*., 2005), SCF (Patella V *et al*., 1998), SCGF beta (Stein A *et al*., 2013), TNF beta (Cassatella MA *et al*., 1993), TRAIL (Collison A *et al*., 2009; Song S *et al*., 2011;), IL-12p40 (Croxford AL *et al*., 2014; Chowdhury IH *et al*., 2014), and IL-2R alpha (Won HY *et al*., 2010).

3.2.1.1.   Interleukin-1alpha (IL-1alpha)

Fig. 1 shows mean plasma levels of IL-1alpha in control, CD and DCM groups. The mean IL-1alpha concentration in control subjects was measured to be 14.63±3.847 pg/mL. As shown, there are slight, insignificant increases in plasma concentrations of IL-1alpha in CD 0 (15.38±2.889 pg/mL) and CD I-II (16.19±4.152 pg/mL) groups when compared to control. On the other hand, mean plasma concentration of IL-1alpha is decreased in CD patients with

NYHA III-IV (12.71±3.570 pg/mL) compared with control group but this is again insignificant. Mean plasma levels of IL-1alpha in DCM patients with NYHA I-II and NYHA III-IV do not differ much from that in control subjects.
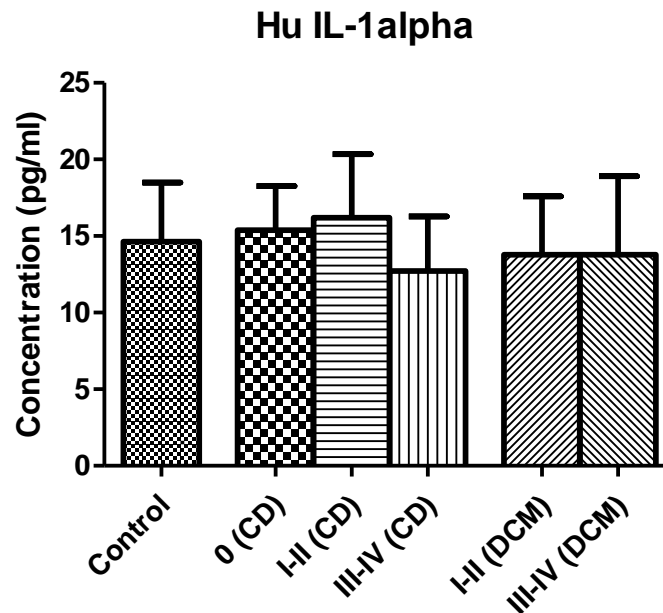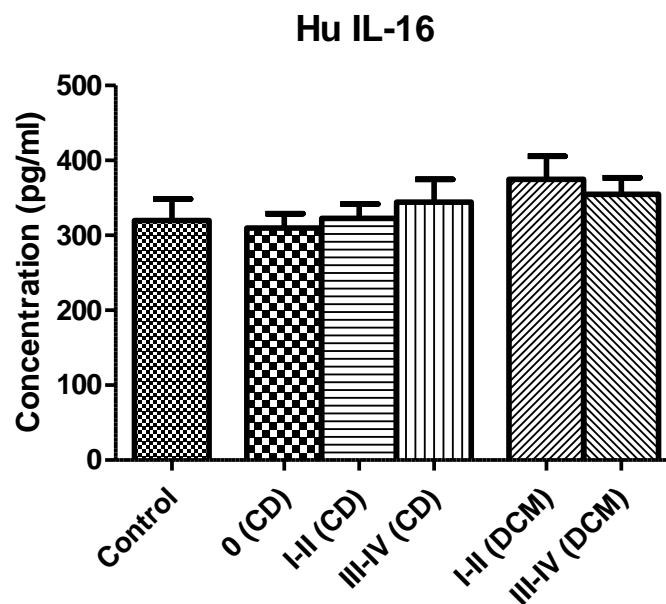

Figure 1. Plasma concentration of IL-1alpha in controls (n = 18); in patients with CD divided in asymptomatic (0) (n = 35), NYHA classes I-II (n = 18), and NYHA classes III-IV (n = 19); and in patients with DCM divided in NYHA classes I-II (n = 11) and NYHA classes III-IV (n = 15). Data is given as mean ± SEM. Hu IL-1alpha, Human interleukin-1 alpha.

3.2.1.2.   Interleukin-16 (IL-16)

Figure 2. Plasma concentration of IL-16 in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 25), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM.

Mean plasma IL-16 concentration in control group was measured to be 319.6±28.94 pg/mL. As shown in **fig. 2**, there is no significant alteration in plasma concentration of IL-16 in CD patients when compared to the control population. There is a slight elevation in mean plasma level of IL-16 in DCM I-II group (374.9±30.76 pg/mL) but this is again statistically not significant.

3.2.1.3.   Interleukin-18 (IL-18)

Fig. 3 below does not demonstrate any significant trend in variation of plasma IL-18 levels in either CD or DCM patients. Although there is an increase in mean IL-18 concentration in CD patients with NYHA III-IV (171.2±28.60 pg/mL) compared to control population (139.1±22.19 pg/mL), and a further increase is also observed in DCM patients with NYHA I-II (182.9±31.81 pg/mL) compared to CD III-IV group, all these changes are statistically insignificant.
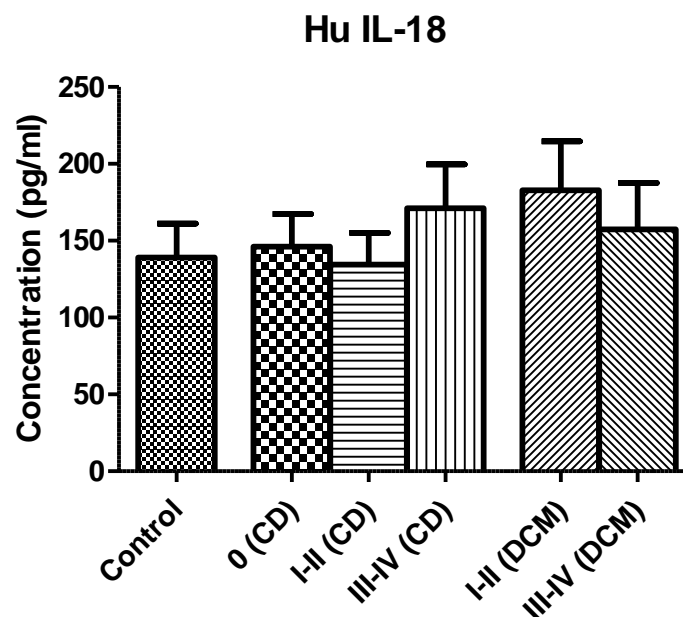

Figure 3. Plasma concentration of IL-18 in controls (n = 34); in patients with CD distributed in asymptomatic (0) (n = 43), NYHA classes I-II (n = 24), and NYHA classes III-IV (n = 27); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 24). Data is given as mean ± SEM.

3.2.1.4.   Macrophage-colony stimulating factor (M-CSF)

There is a visible pattern of increase in mean plasma M-CSF concentration in CD patients as we move from asymptomatic CD 0 group (24.71 ± 4.285 pg/mL) towards CD III-IV group (35.55 ± 6.818 pg/mL), but it still does not represent a significant change. On the other hand, mean plasma levels of M-CSF in DCM patients remain unaltered compared to control group (28.02 ± 5.254 pg/mL).
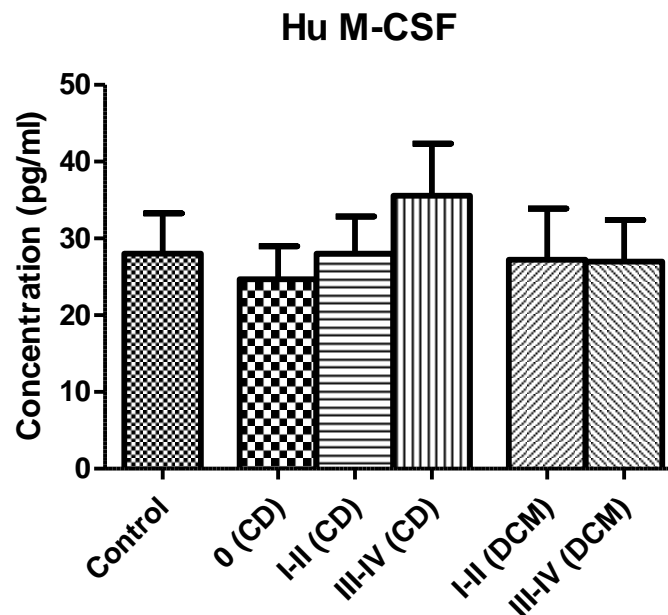

Figure 4. Plasma concentration of M-CSF in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 25), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM.

3.2.1.5.   Macrophage migration inhibitory factor (MIF)

The average concentration of MIF in the control group is 162.9 ± 19.17 (pg/mL). As shown in **fig. 5**, mean plasma levels of MIF are higher in CD patients compared with the control population. In fact, there is a significant increase in MIF levels in CD patients with NYHA III-IV (324.5 ± 64.53 pg/mL) compared to control population ($p < 0.05$). On the other hand, there is a significant decrease in MIF levels in DCM patients with NYHA I-II (171.0 ± 21.61 pg/mL) in comparison to CD patients with NYHA III-IV ($p < 0.05$).
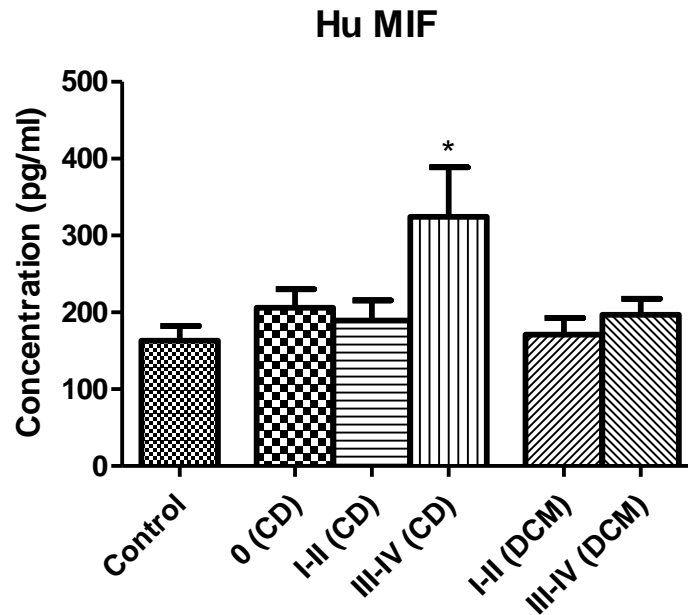
Hu MIF

Figure 5. Plasma concentration of MIF in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 24), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM. * $p < 0.05$ vs control.
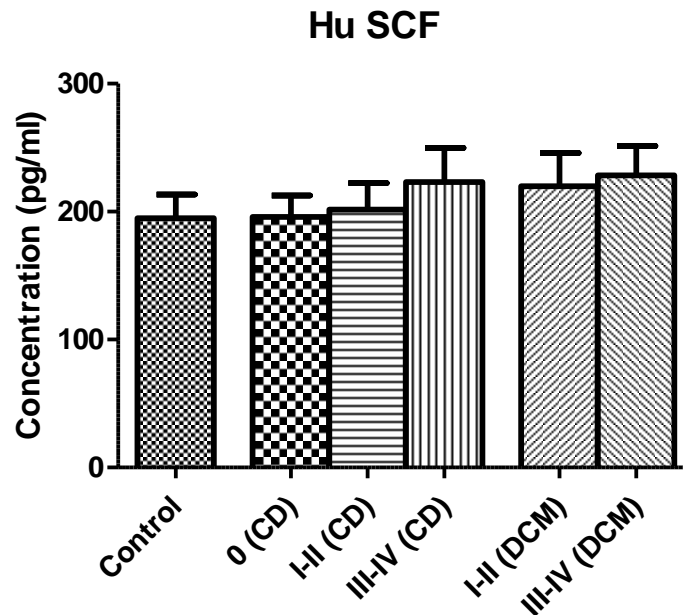
3.2.1.6.   Stem cell factor (SCF)

Hu SCF

Figure 6. Plasma concentration of SCF in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 25), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM.

The mean plasma SCF concentration in control group is 195.0±18.49 pg/mL. The average concentration of SCF in asymptomatic patients (CD 0) remains unaltered compared to control population. Patients in CD III-IV group show a more visible increase in plasma SCF levels (223.1±26.91 pg/mL) compared to control. Similarly, there is also an increase in plasma SCF concentration in DCM patients with NYHA I-II (219.9±26.05 pg/mL) and NYHA III-IV (228.4±23.00 pg/mL), but no statistical difference is detected in any of the above groups.

3.2.1.7.   Stem cell growth factor beta (SCGF beta)

The average concentration of SCGF beta in control population is 24,130±4,856 pg/mL. Asymptomatic CD patients are characterized by unaltered SCGF beta levels (22,943±2,638 pg/mL). Although SCGF beta levels tend to rise in CD and DCM patients with NYHA classes I-II, no statistical difference is detected in any of the groups. However, there is a significant increase in plasma SCGF beta concentrations in both CD and DCM patients with NYHA classes III-IV in comparison to CD patients that are asymptomatic ($p < 0.05$).
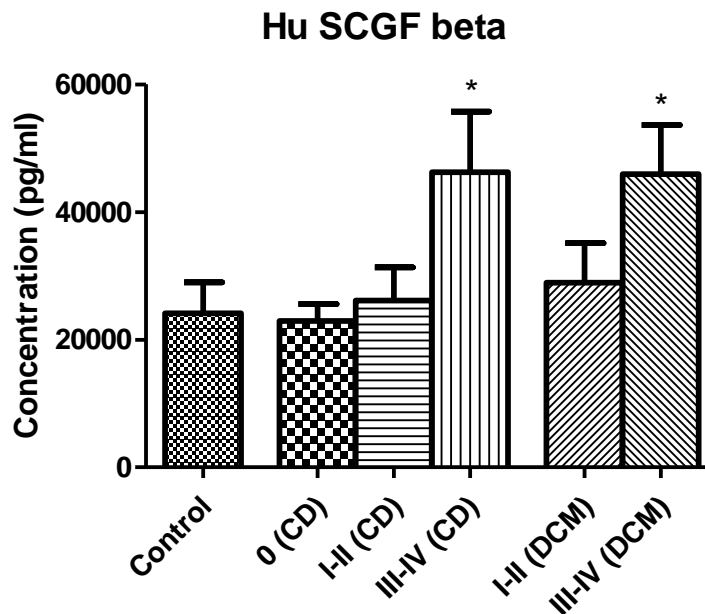

Figure 7. Plasma concentration of SCGF beta in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 25), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM. * $p < 0.05$ vs CD 0.

3.2.1.8.   Tumour necrosis factor beta (TNF beta)

Fig. 8 shows a decrease in TNF beta levels in CD patients when compared to control, although no statistical difference is reported. The average TNF beta concentration in control group is 17.04±3.293 pg/mL. In DCM patients with NYHA I-II (18.48±3.788 pg/mL), the mean plasma TNF beta concentration is slightly higher than in control and CD groups. Further, there is a decrease in TNF beta levels in DCM III-IV group (14.62±4.146 pg/mL) compared to DCM I-II.
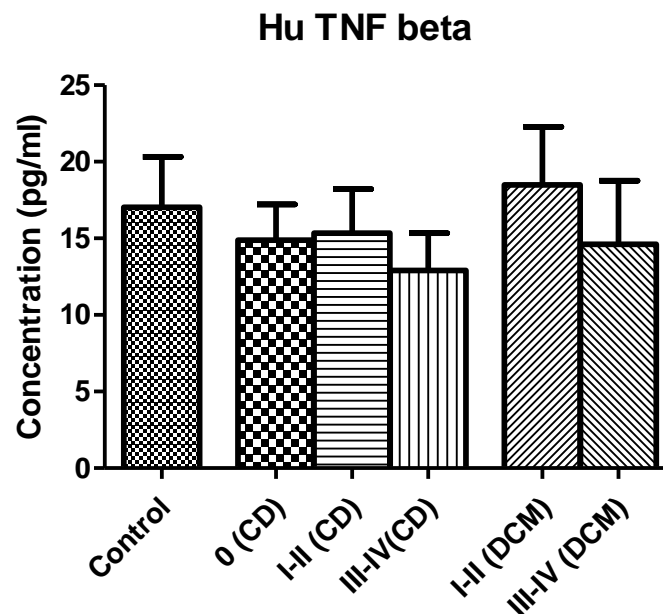

Figure 8. Plasma concentration of TNF beta in controls (n = 14); in patients with CD distributed in asymptomatic (0) (n = 36), NYHA classes I-II (n = 19), and NYHA classes III-IV (n = 17); and in patients with DCM divided in NYHA classes I-II (n = 11) and NYHA classes III-IV (n = 19). Data is given as mean ± SEM.

3.2.1.9.   TNF-related apoptosis inducing ligand (TRAIL)

As shown in **fig. 9**, the mean plasma TRAIL levels in both CD and DCM patients are higher as compared to in control group (148.0±16.86 pg/mL). Within the CD and DCM groups as well, there is a progressive increase in mean TRAIL concentrations as we move to NYHA classes of higher values. The average concentration of TRAIL in CD III-IV patients is 192.3±26.14 pg/mL, while in DCM III-IV patients it is 186.1±28.47 pg/mL. There is still no statistical significance observed in any of the groups.
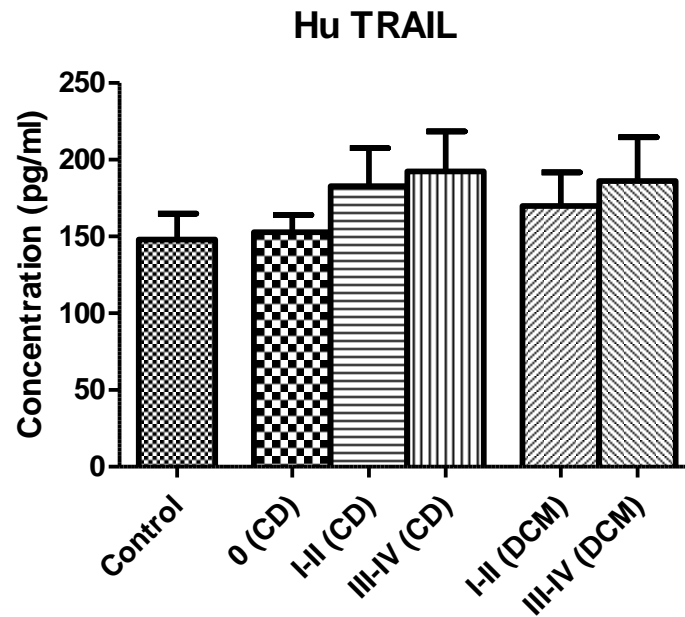
Hu TRAIL

Figure 9. Plasma concentration of TRAIL in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 25), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM.

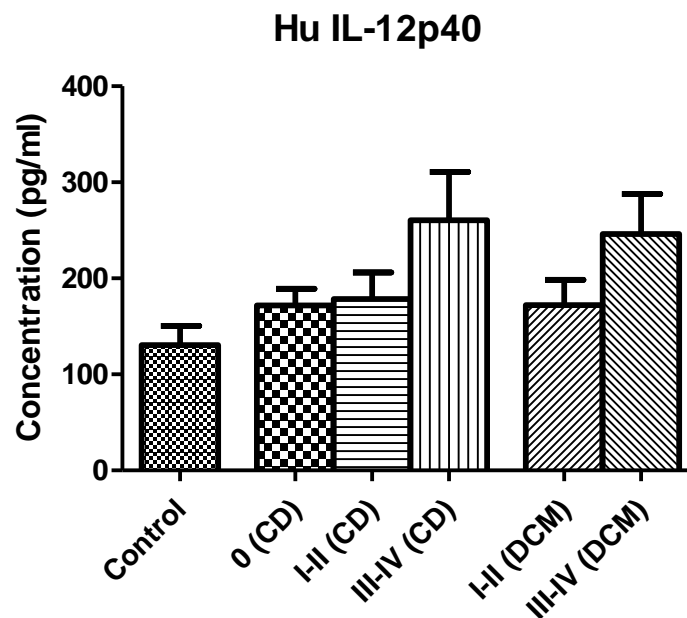### 3.2.1.10.   Interleukin-12p40 (IL-12p40)

Hu IL-12p40

Figure 10. Plasma concentration of IL-12p40 in controls (n = 25); in patients with CD distributed in

asymptomatic (0) (n = 46), NYHA classes I-II (n = 24), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM.

Fig. 10 shows that the mean plasma IL-12p40 concentrations in both CD and DCM patients are higher compared to control population (130.6±19.74 pg/mL). There is a marked increase in IL-12p40 levels in both CD patients with NYHA III-IV (260.3±50.42 pg/mL) and in DCM patients with NYHA III-IV (246.0±41.79 pg/mL) when compared to control group, although no statistical difference is seen in any of the groups.

3.2.1.11. Interleukin-2 receptor alpha (IL-2R alpha)

CD patients without systolic dysfunction are characterized by unaltered plasma levels of IL-2R alpha in comparison to control group (100.9±10.32 pg/mL). However, there is some increase in IL-2R alpha concentration in both CD and DCM patients with NYHA III-IV stages compared to control population. The average concentration of IL-2R alpha in CD III-IV patients is 123.0±15.98 pg/mL, while in DCM III-IV patients it is measured to be 125.1±12.53 pg/mL.
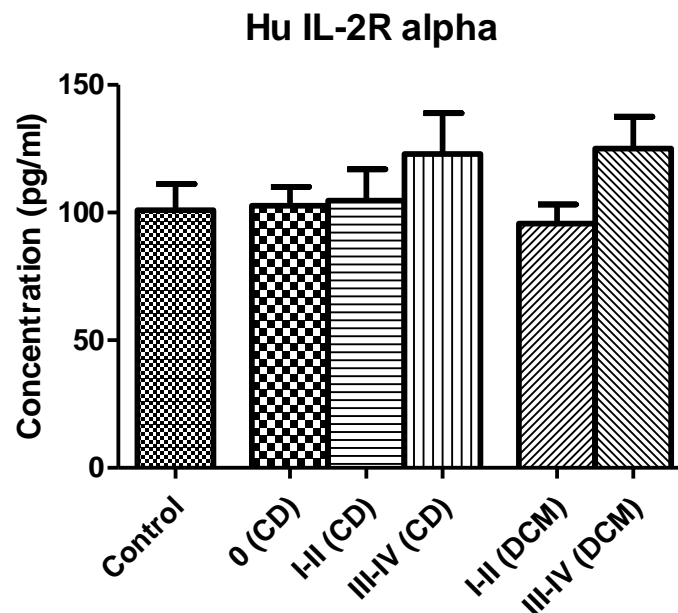

Figure 11. Plasma concentration of IL-2Rα in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 24), and NYHA classes III-IV (n = 23); and in patients

with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM.

3.2.2. Anti-inflammatory cytokines

Two different anti-inflammatory cytokines were investigated in present study: IL-3 (Kovalchin J *et al*., 2010; Srivastava RK *et al*., 2011) and HGF (Mizuno S and Nakamura T *et al*., 2012; Kusunoki H *et al*., 2014).

3.2.2.1. Interleukin-3 (IL-3)

Fig. 12 shows increased plasma concentrations of IL-3 in CD and DCM patients in comparison to control group (73.29±8.889 pg/mL). The IL-3 levels in CD III-IV patients (91.00±10.34 pg/mL) are higher compared to in CD 0 (82.60±7.425 pg/mL) and CD I-II patients (79.53±8.999 pg/mL). Similarly, DCM patients with NYHA III-IV (98.01±9.559 pg/mL) are characterized by increased IL-3 levels in comparison to DCM patients with NYHA I-II (84.69±9.154 pg/mL), but there is no statistical significance relating to any of the groups above.
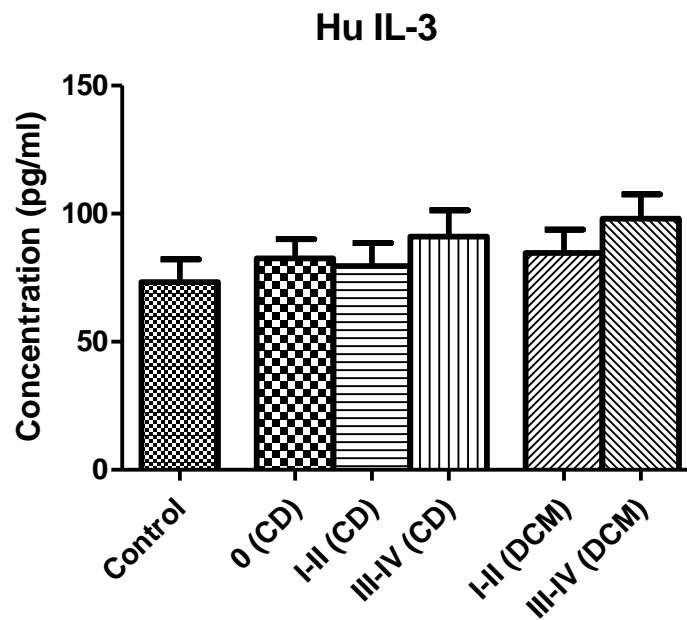

Figure 12. Plasma concentration of IL-3 in controls (n = 24); in patients with CD distributed in asymptomatic (0) (n = 45), NYHA classes I-II (n = 24), and NYHA classes III-IV (n = 22); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM.

3.2.2.2. Hepatocyte growth factor (HGF)

The average concentration of HGF in control population was measured to be 240.2 ± 27.36 pg/mL. Asymptomatic CD patients (242.5 ± 16.88 pg/mL) and those belonging to NYHA class I-II showed unaltered HGF levels compared to control group. However, the plasma levels of HGF were significantly increased in CD and DCM patients with NYHA III-IV when compared to control and asymptomatic CD patients ($p < 0.001$). Mean concentration of HGF in CDIII-IV group was 649.0 ± 91.38 pg/mL, while in DCM III-IV group it was measured to be 550.5 ± 54.94 pg/mL.
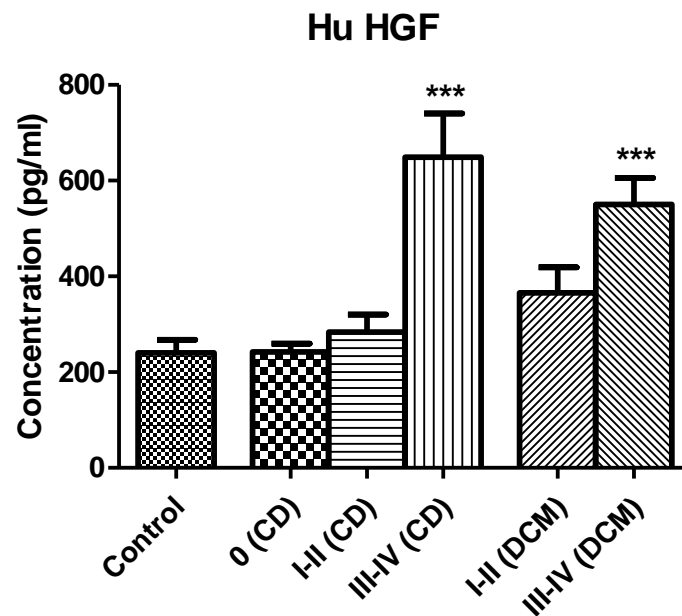

Figure 13. Plasma concentration of HGF in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 25), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM. *** $p < 0.001$ vs control and CD 0.

3.2.3. Cytokines with diverse immune effects

In this study, there were three cytokines with diverse immune effects: IFN-alpha2 (Tarhini AA *et al*., 2012; Levin D *et al*., 2014), beta-NGF (Gaspersic R *et al*., 2010; Peters EM *et al*., 2011; Prencipe G *et al*., 2014) and LIF (Borish L and Rocklin R *et al*., 1992; Alexander H *et al*., 1994).

3.2.3.1. Interferon-alpha2 (IFN-alpha2)

Plasma IFN-alpha2 levels in asymptomatic CD patients and in NYHA class I-II patients remain unaltered when compared to the individuals in control group (57.79±4.081 pg/mL). In CD and DCM patients with NYHA III-IV, there is some increase in IFN-α2 concentrations compared to control but there is no statistical significance detected in any of the groups.
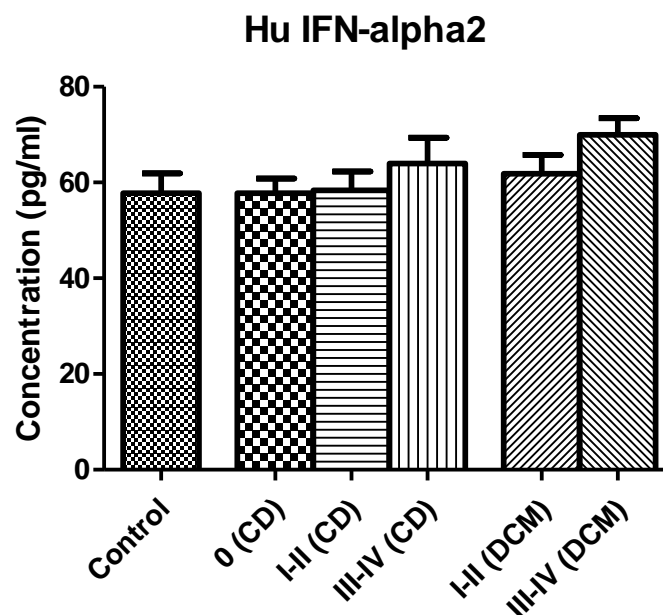

Figure 14. Plasma concentration of IFN-alpha2 in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 45), NYHA classes I-II (n = 24), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM.

3.2.3.2. Beta-nerve growth factor (beta-NGF)

Mean plasma concentration of beta-NGF in control population was measured to be 18.30±4.129 pg/mL. **Fig. 15** shows a steady rise in beta-NGF levels in CD patients as we move from asymptomatic group (16.28±2.615 pg/mL) towards NYHA I-II (19.91±4.375 pg/mL) and then NYHA III-IV (22.51±4.439 pg/mL). Likewise, there is also an increase in beta-NGF levels in DCM patients when we move from NYHA I-II (22.44±5.592 pg/mL) to NYHA III-IV (26.23±5.866 pg/mL), although no statistical difference could be detected in any of the groups.
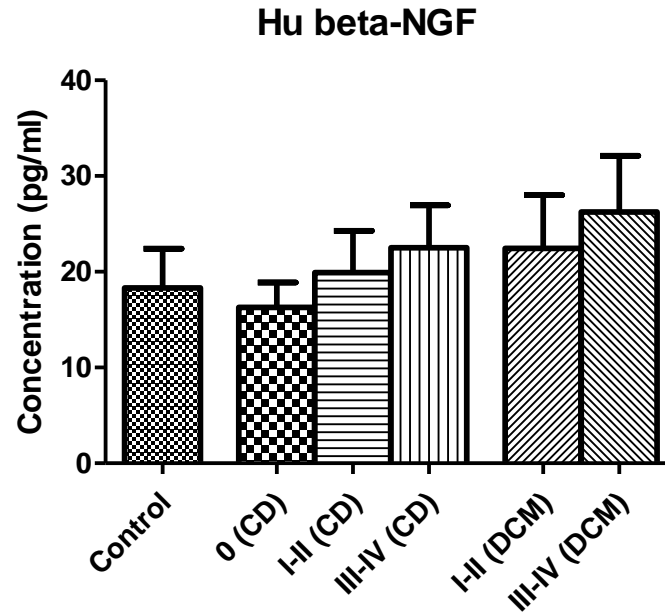
Hu beta-NGF

Figure 15. Plasma concentration of beta-NGF in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 24), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM.

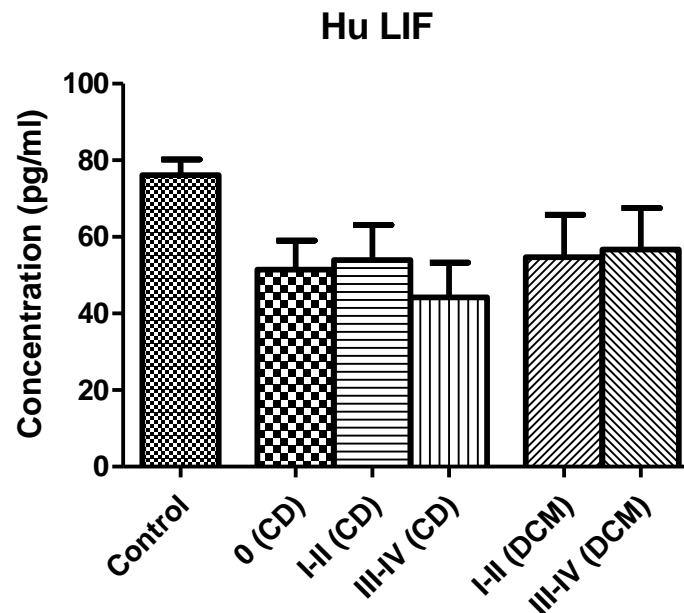### 3.2.3.3. Leukemia inhibitory factor (LIF)

Hu LIF

Figure 16. Plasma concentration of LIF in controls (n = 08); in patients with CD distributed in asymptomatic (0) (n = 20), NYHA classes I-II (n = 15), and NYHA classes III-IV (n = 14); and in patients with DCM divided in NYHA classes I-II (n = 11) and NYHA classes III-IV (n = 12). Data is given as mean ± SEM.

LIF shows a trend which is different from those shown by cytokines mentioned above. In this case as shown in **fig. 16**, mean plasma LIF levels in both CD and DCM patients are decreased compared to control group (76.14±4.100 pg/mL), although no statistical difference is observed in any of the groups. The mean plasma concentration of LIF in CD patients with NYHA III-IV is 44.19±9.091 pg/mL, while in DCM patients with NYHA III-IV it is 56.71±10.79 pg/mL.

3.2.4. Chemokines

Five different chemokines were investigated in present study: CTACK (Meyer N *et al*., 2014; Wang F *et al*., 2014), GRO alpha (Nenseter MS *et al*., 2014; Rohde G *et al*., 2014), MCP-3 (Gonzalez J *et al*., 2013), MIG (Soloski MJ *et al*., 2014; Jovic S *et al*., 2014), and SDF-1alpha (Naqasawa T *et al*., 2014; Karimabad MN and Hassanshahi G *et al*., 2014).

3.2.4.1.   Cutaneous T-cell attracting chemokine (CTACK)

Figure 17. Plasma concentration of CTACK in controls (n = 25); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 25), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM. * *p* < 0.05 vs CD 0.

The average concentration CTACK in control group is 1436±77.27 pg/mL. **Fig. 17** shows a progressive increase in plasma CTACK levels in CD patients as we move from

asymptomatic (0) group (1317±70.09 pg/mL) to CD I-II group (1519±121.4 pg/mL) and then to CDIII-IV group (1798±220.3pg/mL). CD patients with NYHA III-IV particularly show a significant rise in CTACK concentration in comparison to asymptomatic CD patients ($p < 0.05$). Plasma levels of CTACK in DCM patients with NYHA I-II (1504±100.5 pg/mL) and NYHA III-IV (1480±100.5 pg/mL), however, remain unaltered when compared to control group.

3.2.4.2.   Growth related oncogene alpha (GRO alpha)

The mean concentration of GRO alpha in control population is 45.48±8.952 pg/mL. As shown in **fig. 18**, mean GRO alpha levels rise steadily in CD patients as we move from asymptomatic CD (0) group (42.44±6.218 pg/mL) to CD I-II group (48.10±6.131 pg/mL) and then to CD III-IV group (56.01±7.865 pg/mL), although no statistical difference is noted in any of these groups. On the other hand, DCM patients show unaltered GRO alpha levels compared to control population.
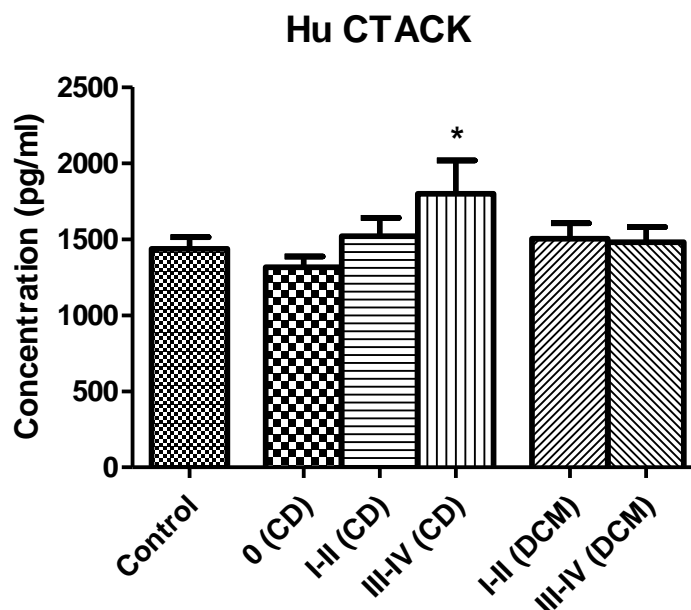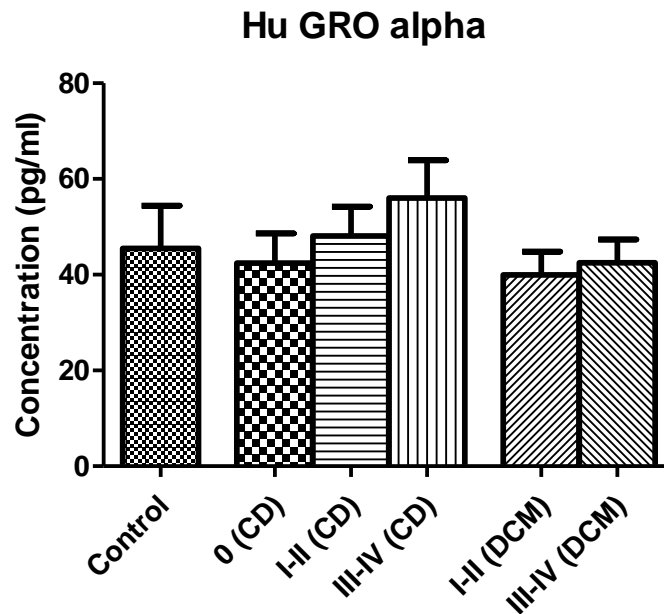

Figure 18. Plasma concentration of GRO alpha in controls (n = 20); in patients with CD distributed in asymptomatic (0) (n = 42), NYHA classes I-II (n = 22), and NYHA classes III-IV (n = 22); and in patients with DCM divided in NYHA classes I-II (n = 17) and NYHA classes III-IV (n = 22). Data is given as mean ± SEM.

3.2.4.3. Monocyte chemo-attractant protein-3 (MCP-3)

The mean concentration of MCP-3 in control group is 19.74 ± 3.743 pg/mL. As shown in **fig. 19**, CD III-IV patients (24.72 ± 4.895 pg/mL) are characterized by increased levels of plasma MCP-3 compared to control, CD 0 group (17.26 ± 1.462 pg/mL) and CD I-II group (15.96 ± 2.099 pg/mL). Similarly, DCM III-IV patients (23.65 ± 3.198 pg/mL) also show raised levels of MCP-3 in comparison to control group and DCM I-II patients (15.75 ± 2.846 pg/mL). However, there is no statistical significance recorded in any of the groups.
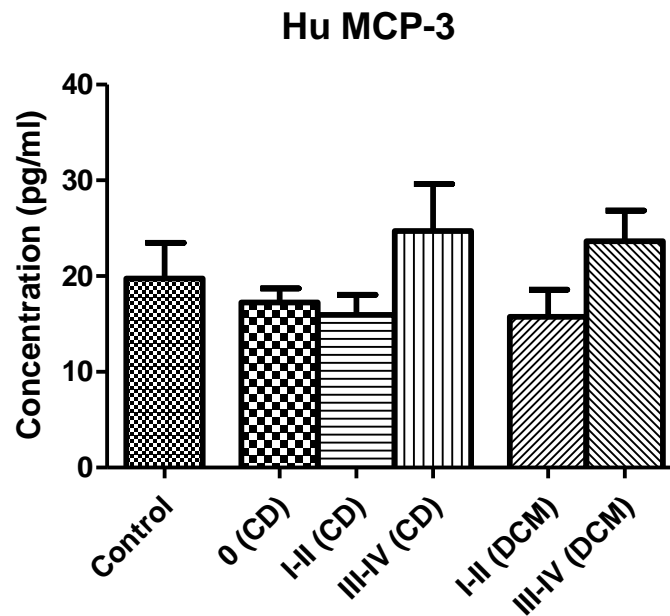

Figure 19. Plasma concentration of MCP-3 in controls (n = 18); in patients with CD distributed in asymptomatic (0) (n = 41), NYHA classes I-II (n = 23), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 20) and NYHA classes III-IV (n = 22). Data is given as mean $\pm$ SEM.

3.2.4.4. Monokine induced by interferon gamma (MIG)

Fig. 20 shows an increase in mean plasma MIG levels in both CD and DCM patients when compared to control population (808.4 ± 147.4 pg/mL). Within the CD group particularly, there is a marked increase in MIG levels as we move across to NYHA classes of higher values. The mean MIG concentration in CD III-IV patients ($2,329 \pm 411.5$ pg/mL) is significantly higher when compared to control group ($p < 0.001$) and asymptomatic CD

group (1224±162.7 pg/mL) ($p < 0.05$). MIG levels in CD III-IV patients are also markedly increased in comparison to DCM I-II patients (832.8±95.29 pg/mL) ($p < 0.01$).

Hu MIG

Figure 20. Plasma concentration of MIG in controls (n = 24); in patients with CD distributed in asymptomatic (0) (n = 46), NYHA classes I-II (n = 25), and NYHA classes III-IV (n = 22); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 25). Data is given as mean ± SEM. ***** *p* < 0.001** vs control; **## *p* < 0.01** vs DCM I-II.

3.2.4.5.   Stromal derived factor-1alpha (SDF-1alpha)
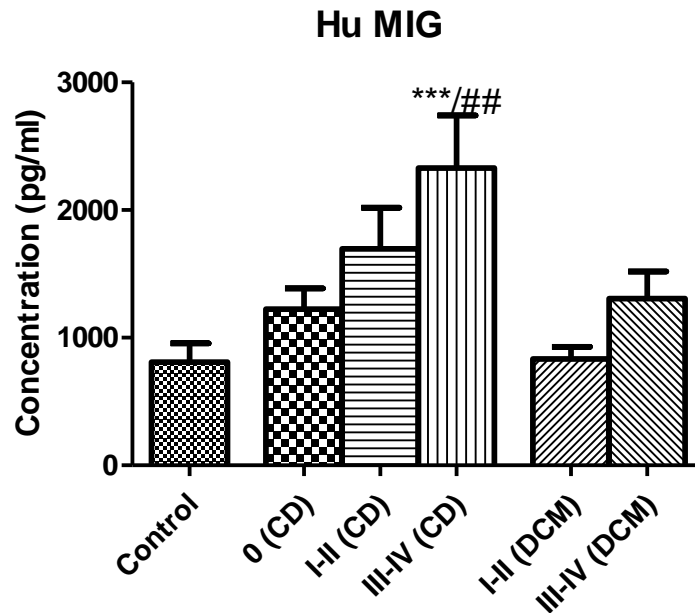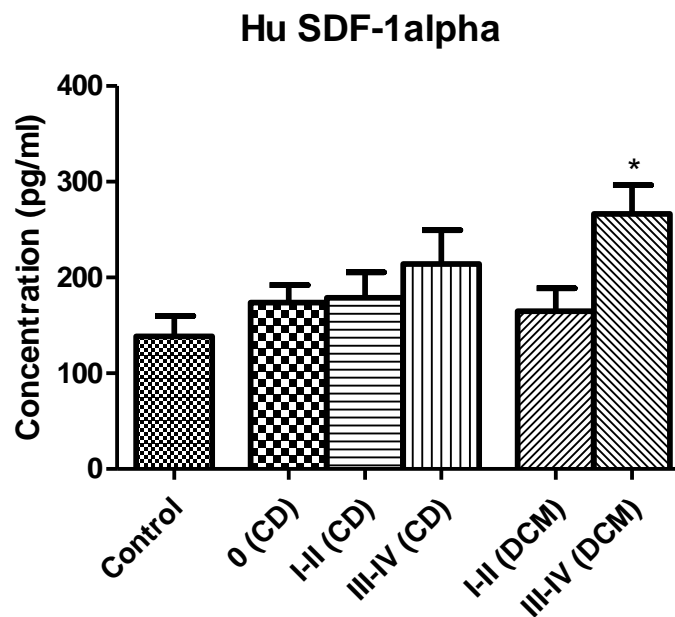
Hu SDF-1alpha

Figure 21. Plasma concentration of SDF-1alpha in controls (n = 21); in patients with CD distributed in asymptomatic (0) (n = 44), NYHA classes I-II (n = 25), and NYHA classes III-IV (n = 23); and in patients with DCM divided in NYHA classes I-II (n = 22) and NYHA classes III-IV (n = 26). Data is given as mean ± SEM. * *p* < 0.05 vs control.

As shown in **fig. 21**, mean SDF-1alpha levels in both CD and DCM patients are higher when compared to control group (138.6±21.32 pg/mL). Here again, there is a rise in SDF-1alpha levels within CD group as we move across to NYHA classes of higher values. The average concentration of SDF-1alpha in asymptomatic CD patients is 173.7±18.30 pg/mL, while in CD patients with NYHA III-IV it is 214.2±35.44 pg/mL. In addition, there is a marked increase in mean SDF-1alpha levels in DCM patients with NYHA III-IV (266.5±29.98 pg/mL) when compared to control (*p* < 0.05).

3.3. Evaluation of predictive and prognostic potency of stem cell growth factor beta

3.3.1. Correlation with echocardiographic parameters

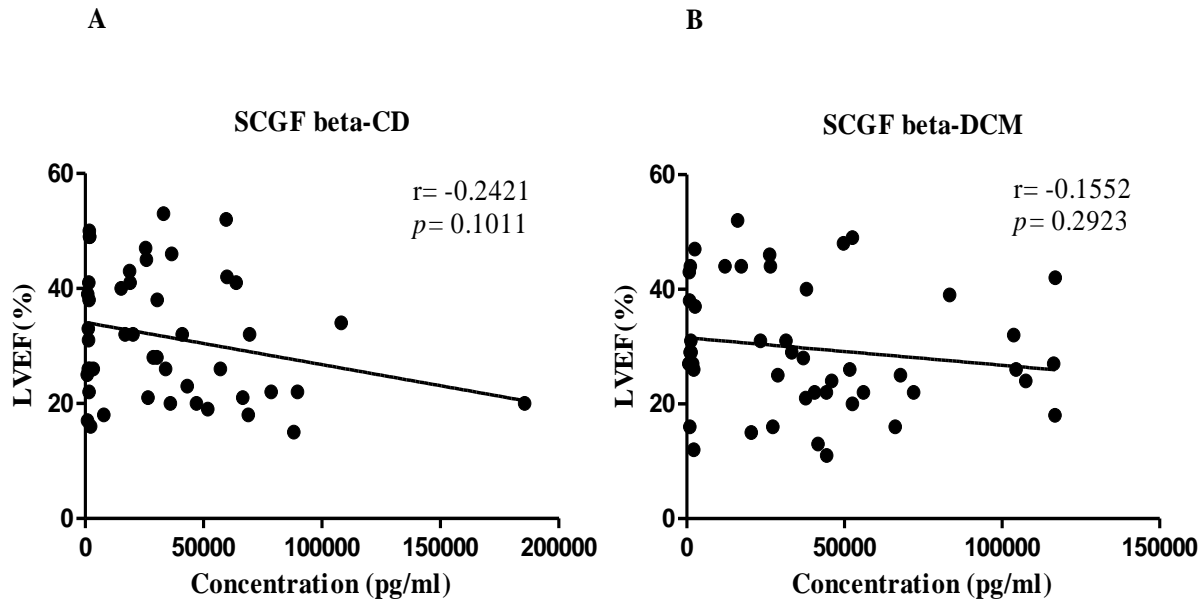**A**   **B**

Figure 22. Plasma SCGF beta concentration correlated to LVEF (%).

A. In patients with CD (n = 47).

B. In patients with DCM (n = 48). LVEF, left ventricular ejection fraction; r, Pearson's correlation coefficient.

	CD (NYHA I-IV)		DCM (NYHA I-IV)	
	r	*p*	**r**	*p*
LVDD	0.0717	0.6357	0.1117	0.4600
LVSD	0.1587	0.2921	0.1362	0.3668
LVEDV	-0.0856	0.5946	0.0065	0.9679
LVESV	0.1707	0.2859	0.0814	0.6129

Table 3. Correlation of plasma SCGF beta concentration to other echocardiographic parameters in CD and DCM patients (LVDD, left ventricular diastolic diameter; LVSD, left ventricular systolic diameter; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume).

A correlation analysis was performed to detect a possible correlation between SCGF beta and LVEF. Pearson's correlation coefficient (r) was used to analyze the correlation between circulating SCGF beta concentration and echocardiograghic parameters in CD and DCM patients (NYHA I-IV). While SCGF beta was found to be significantly increased in CD and DCM patients with advanced HF (NYHA III-IV), its concentration was not significantly correlated with LVEF in patients with CD ($p= 0.1011$) **(Fig. 22A)** and in patients with DCM ($p= 0.2923$) **(Fig. 22B)**. Similarly, a correlation analysis was also performed with other echocardiographic parameters (LVDD, LVSD, LVEDV, and LVESV), but SCGF beta concentration was not found to be significantly correlated to any of these parameters in both CD and DCM groups **(Table 3)**.

3.3.2. Receiver operating characteristic (ROC) and Kaplan Meier curves

Receiver operating characteristic (ROC) curve was generated to find a cut-off value for plasma SCGF beta in CD patients **(Fig. 23A)**. In order to find out whether the SCGF beta cut-off value (15,139 pg/mL) has any prognostic potency for mortality and heart transplantation of patients with CD and DCM, Kaplan-Meier curves were generated using

this cut-off value to divide the patients with CD and patients with DCM into two subgroups. Classical statistics, however, did not visualize any significant predictive value of SCGF beta plasma concentration for risk in lethality or the necessity for heart transplantation in both CD patients (NYHA I-IV) and DCM patients (**Fig. 23B, C**).

A
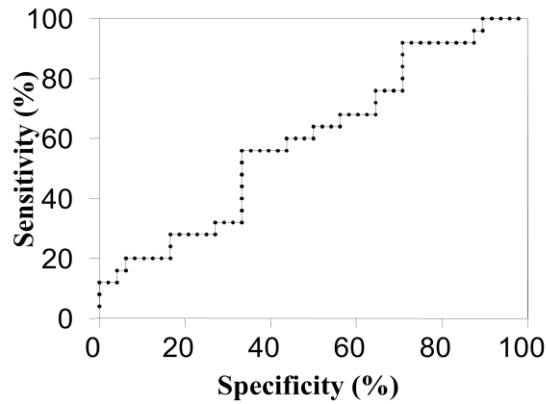
B
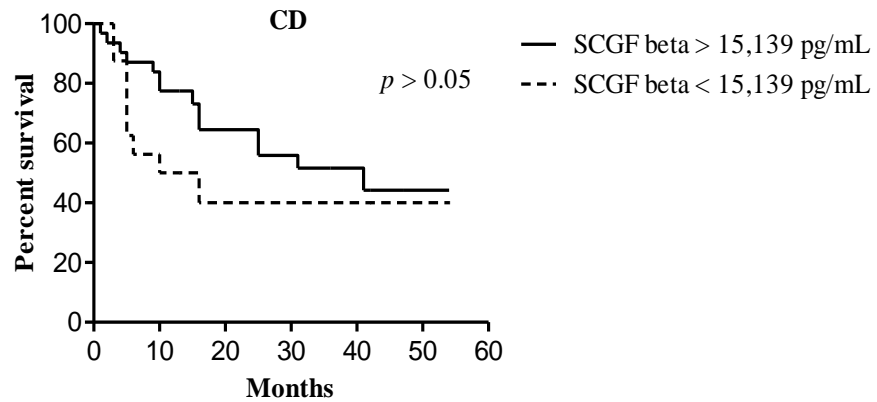
C

Figure 23. Receiver operating characteristic (ROC) and Kaplan-Meier curves (SCGF beta).

A. ROC curve was used to define cut-off value for SCGF beta with best sensitivity and specificity based on CD patients in NYHA classes I-IV (Sensitivity: 56.00%; Specificity: 66.67%). The cut-off value was calculated to be 15,139 pg/mL.

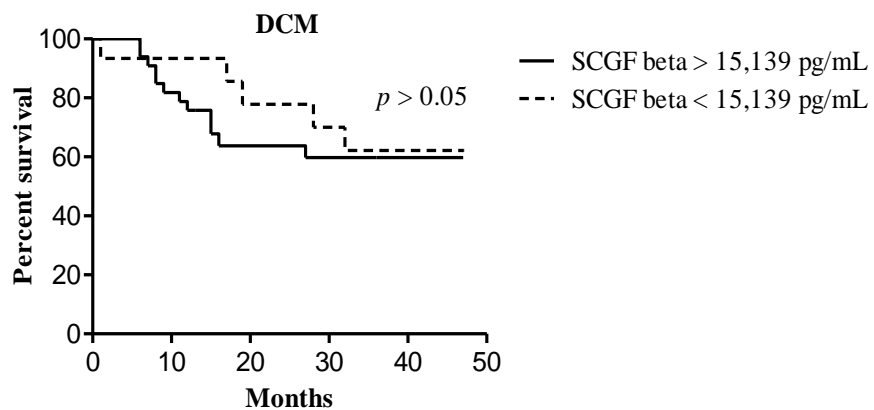**B, C.** Kaplan-Meier survival curves were generated to compare percent survival in CD **(B)** and DCM **(C)** patients with SCGF beta higher or lower than cut-off value (cut-off = 15,139 pg/mL); $p > 0.05$. Statistical analysis was carried out by log-rank test.

3.4. Evaluation of predictive and prognostic potency of hepatocyte growth factor

3.4.1. Correlation with echocardiographic parameters

A

B

HGF-CD

r= -0.4778
p= 0.0007

HGF-DCM

r= -0.2375
p= 0.1041
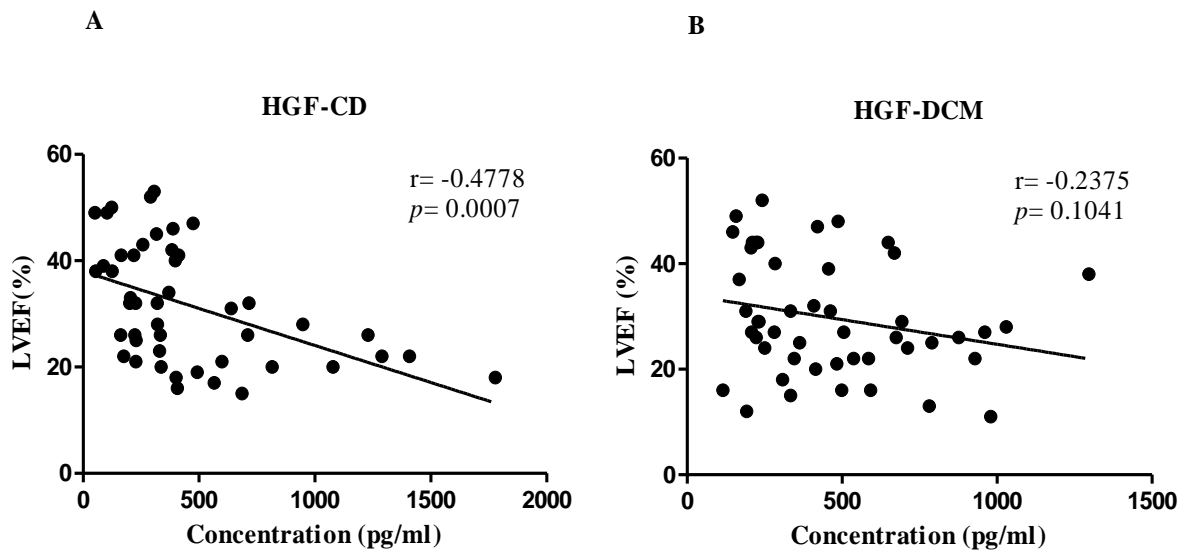
Figure 24. Plasma HGF concentration correlated to LVEF (%).

A. In patients with CD (n = 47).

B. In patients with DCM (n = 48).

As plasma HGF levels were significantly elevated in CD and DCM patients with advanced HF **(Fig. 13)**, a correlation analysis was performed to find out whether the HGF levels were related to impaired cardiac function. While there was a significant correlation between circulating HGF and LVEF in CD patients with NYHA I-IV ($p < 0.001$) **(Fig. 24A)**, there was no correlation in patients with DCM (*p*= 0.1041) **(Fig. 24B)**. Moreover, the data summarized in **table 4** shows that half of the echocardiographic parameters evidenced a correlation with the circulating HGF concentration in patients with CD, with the correlation

reaching significance for the parameters: LVEF (*p*= 0.0007), LVSD (*p*= 0.0167), and LVESV (*p*= 0.0241). However, there was no significant correlation between HGF and any echocardiographic parameters in patients with DCM.

	CD (NYHA I-IV)		DCM (NYHA I-IV)	
	r	*p*	r	*p*
LVDD	0.2215	0.1391	0.1366	0.3652
LVSD	0.3511	0.0167	0.1910	0.2036
LVEDV	0.1357	0.3975	0.2206	0.1657
LVESV	0.3518	0.0241	0.2848	0.0712

Table 4. Correlation of plasma HGF concentration to other echocardiographic parameters in CD and DCM patients.

3.4.2. Receiver operating characteristic (ROC) and Kaplan-Meier curves

A
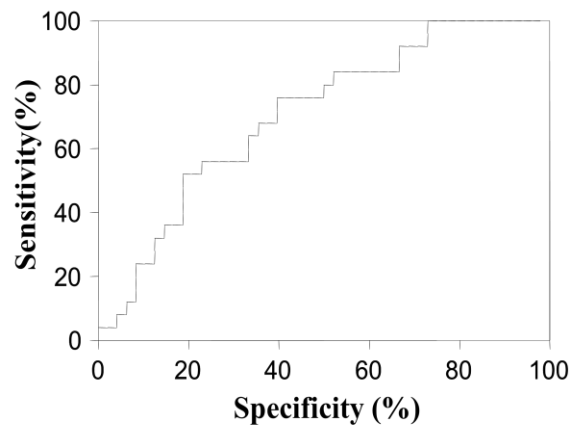
B

C

Figure 25. Receiver operating characteristic (ROC) and Kaplan-Meier curves (HGF).

A. ROC curve was used to define cut-off value for HGF with best sensitivity and specificity based on CD patients in NYHA classes I-IV (Sensitivity: 77.55%; Specificity: 62.22%). The cut-off value was calculated to be 314.3 pg/mL.

B, C. Kaplan-Meier survival curves were generated to compare percent survival in CD **(B)** and DCM **(C)** patients with HGF higher or lower than cut-off value (cut-off = 314.3 pg/mL); $p > 0.05$. Statistical analysis was carried out by log-rank test.

The cut-off value for plasma HGF in CD patients was determined by constructing a ROC curve **(Fig. 25A)**. In order to find out whether the HGF cut-off value (314.3 pg/mL) has any prognostic value for mortality and heart transplantation of patients with CD and DCM, Kaplan-Meier curves were generated using this cut-off value to divide the patients with CD

and patients with DCM into two subgroups. Classical statistics, however, did not visualize any significant predictive value of HGF plasma concentration for risk in lethality or the necessity for heart transplantation in both CD patients (NYHA I-IV) and DCM patients **(Fig. 25B, C)**.

3.5. Evaluation of prognostic and predictive potency of cutaneous T-cell attracting chemokine

3.5.1. Correlation with echocardiographic parameters
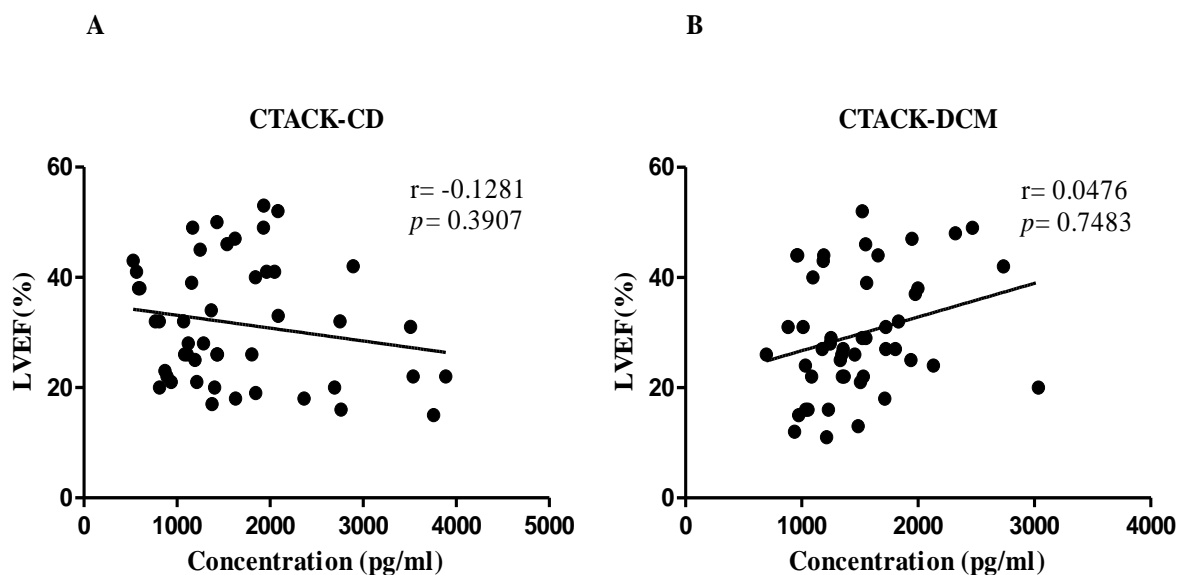
Figure 26. Plasma CTACK concentration correlated to LVEF (%).
A. In patients with CD (n = 47).
B. In patients with DCM (n = 48).

A correlation analysis was performed between plasma CTACK concentration and LVEF. However, it was unable to show any significant correlation between the two parameters in patients with CD ($p= 0.3907$) **(Fig. 26A)** and in patients with DCM ($p= 0.7483$) **(Fig. 26B)**. Further, a correlation analysis was also performed with other echocardiographic parameters like LVDD, LVSD, LVEDV, and LVESV, but plasma CTACK concentration was not found to be significantly correlated to any of these parameters in patients with CD and in patients with DCM **(Table 5)**.

	CD (NYHA I-IV)		DCM (NYHA I-IV)	
	r	*p*	r	*p*
LVDD	0.0243	0.8729	0.0073	0.9616
LVSD	0.0883	0.5595	-0.0179	0.9059
LVEDV	-0.1545	0.3348	-0.1933	0.2259
LVESV	0.0310	0.8476	-0.1337	0.4047

Table 5. Correlation of plasma CTACK concentration to other echocardiographic parameters in CD and DCM patients.

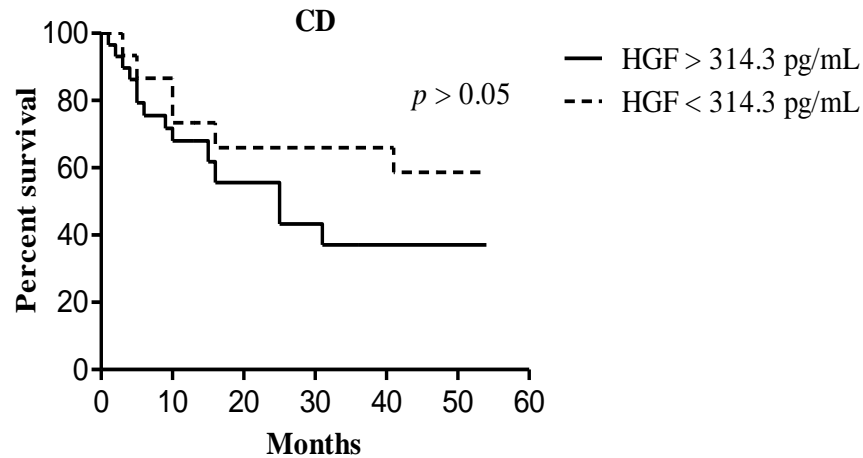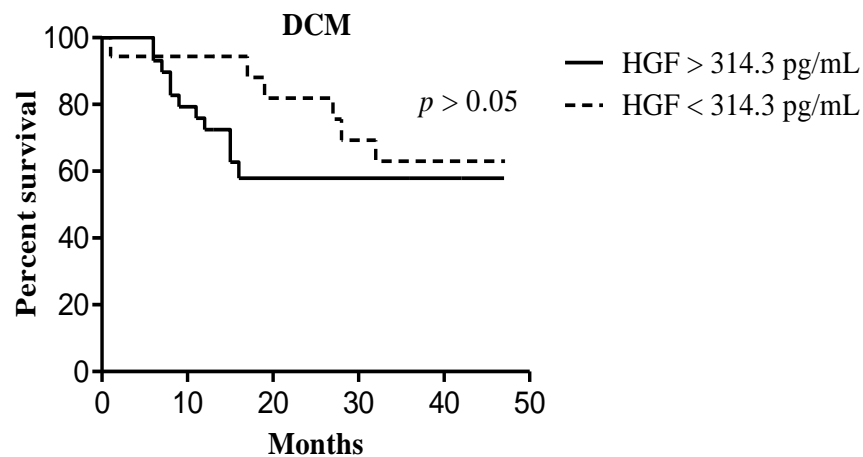## 3.5.2. Receiver operating characteristic (ROC) and Kaplan-Meier curves

A

Figure 27. Receiver operating characteristic (ROC) and Kaplan-Meier curves (CTACK).
A. ROC curve, used to define cut-off value for CTACK with best sensitivity and specificity based on CD patients in NYHA classes I-IV (Sensitivity: 88%; Specificity: 37.5%). The cut-off value was calculated to be 1,803 pg/mL.

B

C
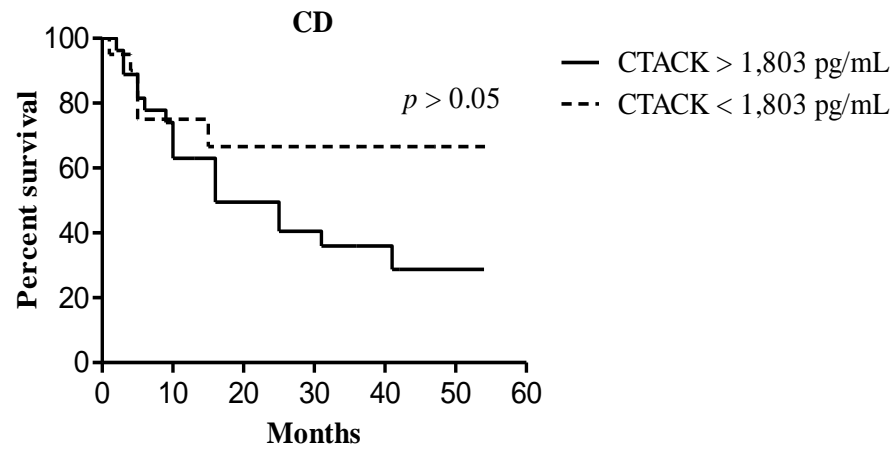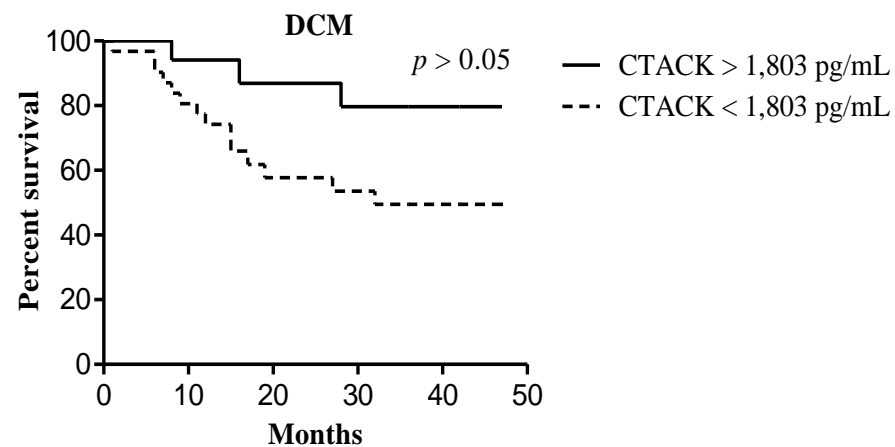
B, C. Kaplan-Meier survival curves were constructed to compare percent survival in CD **(B)** and DCM **(C)** patients with CTACK higher or lower than cut-off value (cutoff = 1,803 pg/mL); $p > 0.05$. Statistical analysis was carried out by log-rank test.

The ROC curve was generated to find a cut-off value for plasma CTACK in CD patients **(Fig. 27A)**. To find out whether the CTACK cut-off value (1,803 pg/mL) has any prognostic potency for the lethality of patients with CD and DCM, Kaplan-Meier curves were generated using this cut-off value to divide the patients with CD and patients with DCM into two subgroups. Classical statistics did not visualize any significant predictive value of CTACK plasma concentration for risk in lethality or the necessity for heart transplantation in both CD patients (NYHA I-IV) and DCM patients **(Fig. 27B, C)**.

3.6. Evaluation of prognostic and predictive potency of monokine induced by interferon gamma

3.6.1. Correlation with echocadiographic parameters

A   **B**
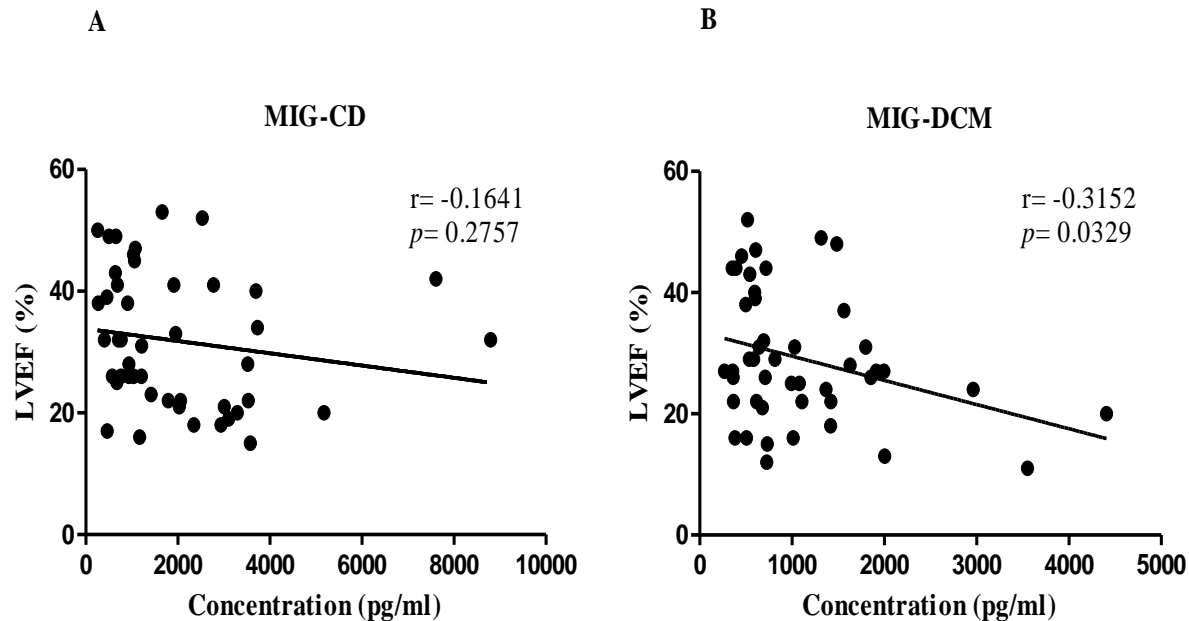
Figure 28. Plasma MIG concentration correlated to LVEF (%).

A. In patients with CD (n = 46).

B. In patients with DCM (n = 46).

A correlation analysis was performed between plasma MIG concentration and LVEF. The analysis failed to reveal any significant correlation between the two parameters in CD patients (p= 0.2757) **(Fig. 28A)**. However, there was a significant correlation detected between plasma MIG concentration and LVEF in patients with DCM (p= 0.0329) **(Fig. 28B)**. A correlation analysis was also done with other echocardiographic parameters such as LVDD, LVSD, LVEDV, and LVESV, but plasma MIG concentration was not found to be significantly correlated to any of these parameters in patients with CD and in patients with DCM **(Table 6)**. Although plasma MIG levels were observed to be significantly increased in CD patients with advanced HF **(Fig. 20)**, no significant correlation was found between MIG and echocardiographic parameters like LVEF in patients with CD.

	CD (NYHA I-IV)		DCM (NYHA I-IV)	
	r	***p***	**r**	***p***
LVDD	0.0699	0.6443	-0.0675	0.6557
LVSD	0.1288	0.3937	-0.1054	0.4857
LVEDV	-0.0233	0.8852	-0.1158	0.4708
LVESV	0.1061	0.5093	-0.1383	0.3885

Table 6. Correlation of plasma MIG concentration to other echocardiographic parameters in CD and DCM patients.
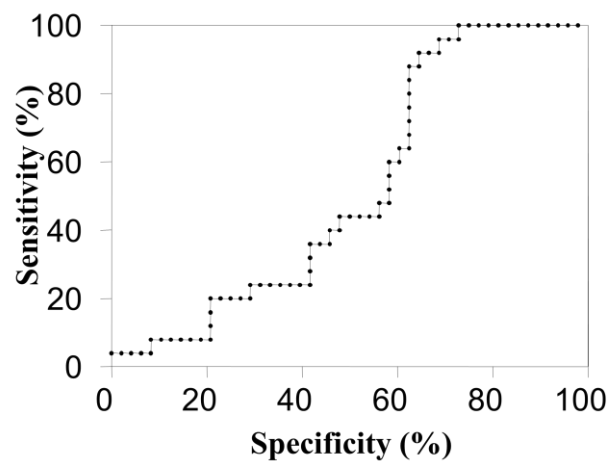
3.6.2. Receiver operating characteristic (ROC) and Kaplan-Meier curves

A
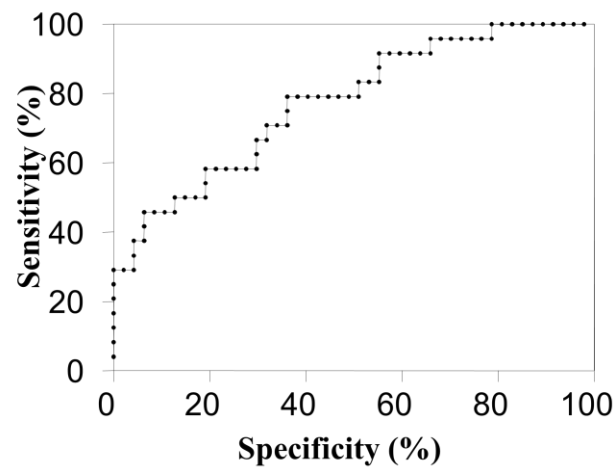
Figure 29. Receiver operating characteristic (ROC) and Kaplan-Meier curves (MIG).
A. ROC curve, used to define cut-off value for MIG with best sensitivity and specificity based on CD patients in NYHA classes I-IV (Sensitivity: 79.17%; Specificity: 63.83%). The cut-off value was calculated to be 1,007 pg/mL.

B
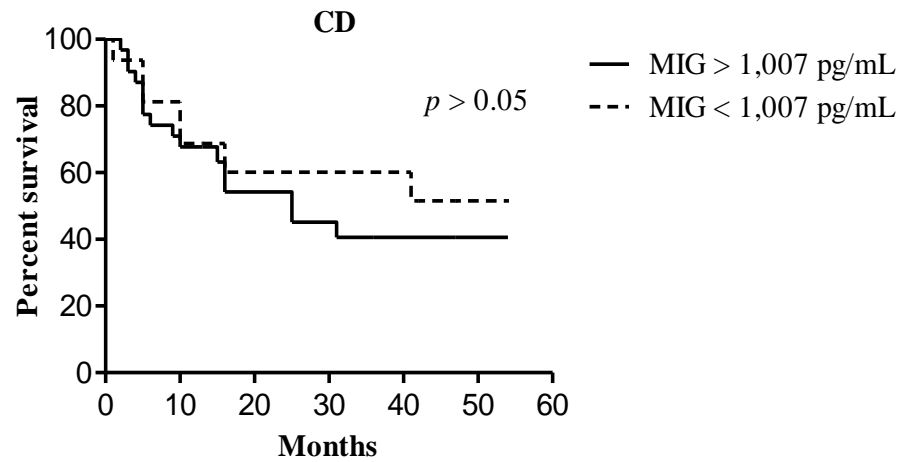
C
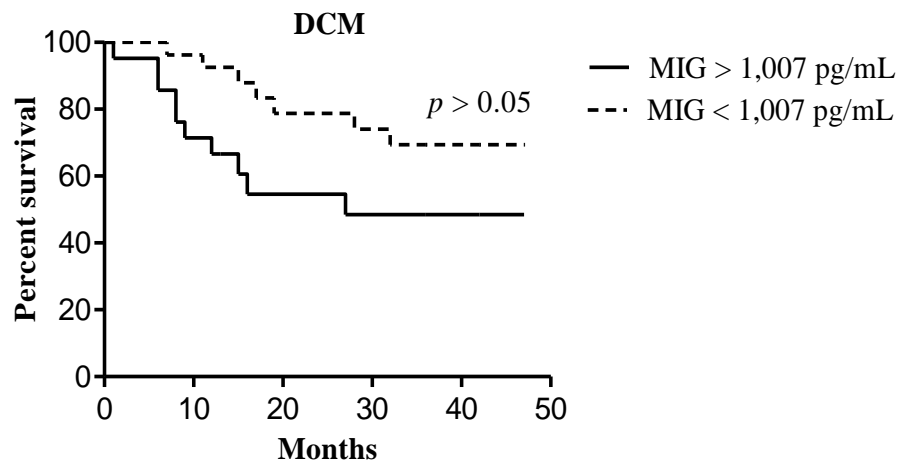
B, C. Kaplan-Meier survival curves were generated to compare percent survival in CD (**B**) and DCM (**C**) patients with MIG higher or lower than cut-off value (cut-off = 1,007 pg/mL); $p > 0.05$. Statistical analysis was carried out by log-rank test.

ROC curve was generated to find a cut-off value for plasma MIG in CD patients (**Fig. 29A**).To find out whether the MIG cut-off value (1,007 pg/mL) has any prognostic potency for mortality and heart transplantation of patients with CD and DCM, Kaplan-Meier curves were generated using this cut-off value to divide the patients with CD and patients with DCM into two subgroups. Classical statistics did not visualize any significant predictive value of MIG plasma concentration for risk in lethality or the necessity for heart transplantation in both CD patients (NYHA I-IV) and DCM patients (**Fig. 29B, C**).

3.7. **Multivariate analysis**By using multivariate analysis, this study tried to investigate whether two or more cytokines together could demonstrate any predictive and prognostic potency in patients with CD and in patients with DCM due to idiopathic causes.

3.7.1. Chagas' disease

Univariately, HGF was the only cytokine that was found to be significant. A forward stepwise discriminant analysis selected HGF and GRO alpha as 'the best' parameters and was able to separate 70.9% (cross-validated sensitivity/specificity of 31.8%/84.4%) survivors from the deceased. When imputing method was applied, more parameters for the multivariate analysis were allowed (when only one of 21cytokinesvalue was missing, then this patient was usually excluded from the discriminant listwise analysis). In the original data analysis, only 40.5% of patients were considered (N = 45). By imputing, this value was increased to 84.7% of patients (N = 94). Multivariately, the following parameters were selected: HGF and IL12p40 with a cross-validated separation of 81.9% (sensitivity/specificity of 92.9%/50.0%), an improvement of 11%.

3.7.2. DCM

Univariately, IL-16 was only found to be significant. In forward stepwise discriminant analysis, IL-16and IL-2R alpha were selected as 'best' parameters and was able to correctly classify 66.7% (cross-validated sensitivity/specificity of 74.2%/52.9%) survivors from the deceased. In the original data analysis, only 32.3% of patients (N = 20) were available. By imputing, it was increased to 77.4% of patients (N = 48). The DCM group now contained almost 80% of complete data. The best multivariate features were SDF-1alpha, SCF, and MIG, cross-validated with a separation of 77.1% (sensitivity/specificity of 76.5%/77.4%), an improvement of more than 10%. Statistically, this is very interesting as none of these three cytokine parameters were found to be significant univariately.

4. Discussion

4.1. Role of inflammation in pathogenesis and progression of heart failure

Chronic HF puts a major burden on public health, and its prognosis can be compared to that of different malignant diseases. HF is characterized by the activation of various inflammatory processes that result in increased levels of inflammatory markers such as IL-6 and CRP (Anker SD and von Haehling S, 2004). This activation may occur due to several reasons such as myocardial damage, reduced cardiac output, and/or hemodynamic overload (Pasic J *et al*., 2003). A vicious circle is present between HF and inflammation as inflammatory markers have been found to be associated with worsening of cardiac function and poor prognosis.

Cytokines belong to a group of relatively low molecular weight, pharmacologically active proteins. Some of the important pro-inflammatory cytokines implicated in the progression of HF are TNF alpha and IL-1. These cytokines are secreted by all nucleated cell types present in the myocardium, including the cardiac myocytes. The growing interest in understanding the role of inflammatory mediators in HF arises from the fact that many aspects of the syndrome of HF can be related to the known biological effects of inflammatory cytokines. When these cytokines are expressed at high concentrations, they are sufficient to mimic some aspects of the so-called heart failure phenotype, including progressive left ventricular (LV) dysfunction, pulmonary edema, LV remodeling, fetal gene expression, and cardiomyopathy. Therefore, the "cytokine hypothesis" for HF states that HF progresses, at least partially, due to the toxic effects of the endogenous cytokine cascades on the heart and the peripheral circulation (Andrade ZA, 1983). It emphasizes on the point that cytokines may not be the initial cause of HF, but rather that the over expression of cytokine cascades contribute to the disease progression of HF. Thus, the expression of cytokines, just like the expression of neurohormones, may represent a biological mechanism that is responsible for worsening HF.

4.2. Role of inflammation in pathogenesis and progression of Chagas' disease

Inflammatory processes mediated by cytokines also play a key role in the pathogenesis of CD. Activation of inflammation in CD differs from that in DCM from idiopathic causes and is associated with increased severity of HF (Mocelin AO *et al*., 2005). In chronic CD patients with cardiomyopathy, there is progressive and persistent inflammation of myocardial fibers that leads to gradual impairment of contractile function and dilatation of all four chambers of the heart. With time, the myocytes are gradually lost due to inflammatory tissue destruction, and the dead myocytes are then replaced by intense fibrosis, which predispose the patient to development of HF, ventricular arrhythmias, and other serious pathologies (Andrade ZA, 1983; Rassi A Jr *et al*., 2009).

Mononuclear cellular infiltrate is present throughout the myocardium with vast areas of confluent fibrosis in CD but is not seen in idiopathic DCM (Rossi MA *et al*., 2003; Nunes VL *et al*., 2006). This widespread myocardial inflammatory infiltrate can lead to a more severe ventricular remodeling process, causing a rapid and severe onset HF, and ultimately death. Patients with HF due to CD, therefore, are associated with a poorer prognosis and outcome in comparison to patients with DCM from idiopathic causes (Pereira Nunes Mdo C *et al*., 2010; Barbosa AP *et al*., 2011).

4.3. Role of cytokines in cardiac remodeling

CD is characterized by progressive cardiac remodeling, which may finally result in DCM and congestive heart failure, the most serious manifestation of chronic CD. Ventricular remodeling includes various processes such as cardiomyocyte loss through necrosis or apoptosis, hypertrophic response of remaining cardiomyocytes, angiogenesis, and an architectural rearrangement of extracellular matrix (Cohn JN *et al*., 2000; Lamblin N *et al*., 2005). Various studies indicate that endogenous repair mechanisms regulated by stem cells are involved in chronic cardiac remodeling. This in turn is coordinated by various types of cytokines, growth factors, and chemokines (Cui Y and Madeddu P, 2011; Fortini C *et al*., 2011). For example, plasma levels of HGF, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and SDF-1alpha are significantly increased in HF

patients as compared to in healthy subjects (Fortini C *et al*., 2011). There is a possibility of using some of the cytokines as prognostic markers in HF patients.

4.4. Inflammatory biomarkers in Chagas' disease

Specific local or systemic pathways of inflammation might be particularly active in patients with cardiomyopathy due to CD, and could be responsible for the variations observed in clinical course of disease, when compared to DCM from other causes (Mocelin AO *et al*., 2005). By identifying such serum biomarkers that might be involved in regulation of these inflammatory pathways, we may be able to use them as tools for early diagnosis and prognosis of CD. As cardiomyopathy due to CD is associated with a poor prognosis, identifying patients accurately at an early stage of the disease may help in providing effective treatment and prevent the development of HF and thus early death.

In this study, 21 different cytokines were investigated in both CD and idiopathic DCM patients. The serum levels of each of these cytokines were measured in both groups of patients to see if they were regulated and whether there was any difference in regulation from one group to the other. Out of the 21 different inflammatory cytokines that were investigated, 10 of them have been found to be regulated in patients with HF from various causes, according to the present literature. These include IL-1alpha (Munger MA *et al*., 1996), IL-18 (von Haehling S *et al*., 2009), M-CSF (Hohensinner PJ *et al*., 2010), MIF (Liu Y *et al*., 2003), TRAIL (Niessner A *et al*., 2009), HGF (Rychli K *et al*., 2011), beta-NGF (Kaye DM *et al*., 2000), LIF (Hirota H *et al*., 2004), GRO alpha (Damas JK *et al*., 2000),and SDF-1alpha (Fortini C *et al*., 2011).

This investigation demonstrated a number of cytokines, whose mean plasma concentrations were significantly increased in CD patients with advanced HF (NYHA III-IV) when compared to healthy subjects. These included: MIF, SCGF beta, HGF, CTACK, and MIG. In addition, cytokines like SCGF beta, HGF, and SDF-1alpha also showed significant elevation in plasma levels in DCM patients with advanced HF (NYHA III-IV) in comparison to control group. However, none of these investigated cytokines were able to show any prognostic potency in predicting mortality or necessity for heart transplantation in both CD patients and idiopathic DCM patients.

4.4.1. Role of hepatocyte growth factor as a serum biomarker in heart failure

HGF was found to be significantly increased in plasma of patients with advanced HF (NYHA III-IV) in both CD and idiopathic DCM groups, but not in patients with HF of mild to moderate severity (NYHA I-II). However, HGF concentration in HF patients was lower than the values previously reported (Lamblin N *et al*., 2005; Rychli K *et al*., 2011). One reason for this difference in values could be the different methods used for cytokine measurement. In this study, Bio-Plex Pro™ Assays system (Bio-Rad) was used, while in the previous two studies, traditional ELISA kit for HGF was used. However, the most probable reason could be the different composition of recruited patients and the treatment they received. It has been confirmed through various clinical trials that HGF concentration is positively correlated with age and clinical severity (Lamblin N *et al*., 2005; Rychli K *et al*., 2011). In one of the trials (Rychli K *et al*., 2011), the median age of recruited patients is even 75 years and only advanced HF patients were included. On the other hand, the average age of HF patients in present study is only 49 years and patients from NYHA classes I-IV were included.

Plasma HGF concentration may also have been influenced by the difference in treatment received by HF patients. In a previous study, only hospitalized HF patients with clinical signs and symptoms of cardiac decompensation (NYHA III-IV) were included, and blood was collected on the morning of discharge before the intake of medication (Rychli K *et al*., 2011). In another study, peripheral blood was collected for HGF measurement at the time of entry into the study (Lamblin N *et al*., 2005). However, in this present study, the heart function of all patients was compensated and they were treated with maximal tolerated doses of ACEIs, ARBs, and other drugs, for at least three months before the blood was obtained. Since the dose of ACEIs has been found to be inversely correlated with HGF concentration (Rychli K *et al*., 2011) and most of the patients in present study received ACEI therapy, this may also be one of the reasons why the HGF concentration measured was lower than previously described.

HGF is a cytokine that has been shown to be a strong and independent predictor of mortality in patients with advanced HF. However, this association is only present in patients with ischemic HF and not in those with HF from non-ischemic causes like CD. This may suggest the presence of discrete pathogenic pathways regulating the course of disease (Rychli K *et*

al., 2011). Serum HGF levels seem to be regulated mainly in ischemic conditions of the heart. This is supported by the finding that HGF is released into the circulation after myocardial infarction (Zhu Y *et al*., 2000). HGF, which was originally identified as a potent mitogen for hepatocytes, is now known to have mitogenic, anti-apoptotic, angiogenic, and anti-fibroblast effects in various cell types in the body (Matsumoto K and Nakamura T, 1996). The endogenous HGF system of the body protects the heart via its anti-apoptotic effect on cardiomyocytes and attenuates the development of HF with increased angiogenesis and decreased fibrosis and apoptosis (Lamblin N *et al*., 2005). HGF is known to be a growth factor for vascular tissues, and it has the most potent mitogenic activity among various known growth factors (Nakamura Y *et al*., 1996). Therefore, an increase in serum HGF concentration after an episode of acute myocardial infarction may contribute to the formation of new collateral blood vessels around the ischemic area (Zhu Y *et al*., 2000). In this study, patients with coronary artery diseases were excluded. Therefore, there were no cases of HF from ischemic causes in present study.

It is also interesting to note that echocardiographic parameters have a close correlation with plasma HGF concentration in CD patients but not in patients with idiopathic DCM, despite the fact that CD leads to typical DCM. LVEF, for instance, is negatively correlated with plasma HGF levels in CD patients ($p= 0.0009$), but there is no strong correlation with HGF concentration in DCM patients ($p= 0.1041$). It would be very interesting if DCM due to specific etiology may have an effect on the generation and metabolism of HGF, and subsequently on the concentration of circulating HGF. If this can be proven, it might change our general understanding of DCM, since it is generally believed that the phenotype of DCM itself and not the causes of the phenotype determine the regulation of biomarkers of interest (Wang Y *et al*., 2012). Therefore, further investigations with more patients per specific etiology are required in order to clarify these mechanisms.

4.4.2. Macrophage migration inhibitory factor as an inflammatory biomarker in heart failure

MIF is another example of such a cytokine that is regulated in HF cases due to specific etiologies. Plasma MIF concentration has been found to be increased in patients with congestive heart failure, and interestingly the increase was more significant in cases of coronary artery disease as compared to the increase in levels observed in cases of DCM.

MIF is a pro-inflammatory cytokine which may be involved in inflammatory processes related to HF due to ischemic processes (Liu Y *et al*., 2003). Inflammation may be more severe in ischemic myocardium as compared to in DCM. In present study, mean plasma MIF concentration in patients with advanced HF (NYHA III-IV) due to CD (324.5±64.53 pg/mL) was higher than the mean MIF levels in advanced HF patients with idiopathic DCM (196.9±20.72 pg/mL). Here again, it might indicate that inflammatory processes involved in the pathogenesis and progression of CD may be more severe and pronounced than in idiopathic DCM (Rossi MA *et al*., 2003; Nunes VL *et al*., 2006).

4.4.3. Regulation of stem cell growth factor beta in heart failure – potential as a biomarker

There were some cytokines in this study whose role in HF from various causes remains unknown and undefined. SCGF beta, for instance, had never been reported in HF patients prior to present study. SCGF is a novel human growth factor for hematopoietic progenitor cells. Two isoforms (alpha and beta) have been identified in humans. SCGF beta is 78 amino acids shorter than SCGF alpha (Hiraoka A *et al*., 1997; Mio H *et al*., 1998). Both isoforms are bioactive and expressed in a similar way. SCGF beta exhibits burst-promoting activity and granulocyte/macrophage colony-stimulating activity on erythroid and granulocyte/macrophage progenitor cells in combination with other cytokines. Its concentration in serum is also known to increase following stem cell transplantation in patients (Ito C *et al*., 2003).

In present study, SCGF beta was found to be significantly increased in plasma of advanced HF patients (NYHA III-IV) caused by CD and idiopathic DCM, as compared CD patients without HF. However, despite this significant increase in advanced HF patients, plasma SCGF beta concentration had no significant correlation with echocardiographic parameters. Similarly, Kaplan-Meier curves failed to visualize any significant predictive power of plasma SCGF beta concentration for risk in lethality or the necessity for heart transplantation in both CD and DCM patients.

In recent times, it has been recognized that the endogenous bone marrow-cardiac axis may also play an important role in myocardial repair and functional recovery of patients with congestive heart failure. Hematopoietic stem and progenitor cells, especially endothelial progenitor cells (EPC), have been identified to be not only potential biomarkers for cardiac

events but also as therapeutic targets. There is a progressive decrease of circulating EPC with increasing severity of HF (Maltais S *et al.*, 2011). On the other hand, mesenchymal stem cells and subset of hematopoietic stem cells were increased significantly at specific stages of HF. A number of investigated cytokines as mentioned above were also increased in HF patients (Fortini C *et al.*, 2011). Therefore, corresponding cytokines and/or growth factors are needed for proliferation, mobilization, homing, and local function of bone marrow-derived stem or progenitor cells under HF condition, in order to regulate and sustain these processes.

Serum SCGF beta levels are known to be raised after stem cell transplantation (Ito C *et al.*, 2003). Mobilized bone marrow cells that move towards the failing heart may influence local inflammatory processes and promote cardiac regeneration. This process may require SCGF beta. However, nothing has been reported about its concentration in HF patients. This study is the first to demonstrate SCGF beta in HF patients. Although there was a significant elevation in circulating SCGF beta levels in advanced HF patients in both CD and DCM groups, it had no correlation with LVEF and thus cannot be used as a prognostic marker for mortality and heart transplantation. Further studies need to be done in order to clarify the mechanism governing the SCGF beta regulation in advanced HF patients and the function such elevation might have.

4.4.4. Possible role of monokine induced by interferon gamma as a serum biomarker in Chagas' disease

MIG is another cytokine whose role in patients with HF is unknown. In this study, plasma MIG concentration was significantly elevated in CD patients with advanced HF (NYHA III-IV) (2,329 pg/mL) as compared with control group (808.4 pg/mL) ($p < 0.001$). Furthermore, circulating levels of MIG in CD patients with HF were considerably higher than the levels obtained in DCM patients with HF, although no statistical difference was observed in this case. Mean MIG concentration in CD patients (NYHA I-II) was 1695 pg/mL, more than twice the mean MIG levels recorded in DCM patients (NYHA I-II) (832.8 pg/mL). Similarly, in advanced HF patients with CD (NYHA III-IV) (2,329 pg/mL), mean MIG concentration was considerably higher than in DCM patients with advanced HF (NYHA III-IV) (1,307 pg/mL). Even the mean plasma MIG level in CD patients with mild to moderate

HF (NYHA I-II) (1695 pg/mL) was recorded to be more than twice the mean MIG concentration observed in control subjects (808.4 pg/mL).

Although circulating MIG concentration in CD patients with HF had no significant correlation with echocardiographic parameters like LVEF, significant correlation of plasma MIG levels with LVEF was found in DCM patients (p= 0.033). Correlation of LVEF in both CD and DCM groups was negative (MIG concentration increased with decrease in ejection fraction). On the other hand, Kaplan-Meier curves were unable to show any significant predictive power of MIG for risk in mortality or necessity for heart transplantation in both CD and DCM patients.

MIG is a chemokine, which acts as a signaling molecule, regulating the movement of immune cells, directing them to sites of tissue injury and inflammation and modulating their states of activation and effecter cell function (Soloski MJ *et al*., 2014). MIG has been found to increase experimentally in mice with HF, although it is etiology dependent (Vistnes M *et al*., 2010). MIG has also been studied in experimental mice models infected with *T.cruzi*. It has been shown in these studies that MIG and other interferon gamma-inducible factors such as VCAM-1 (vascular cell adhesion molecule-1), and chemokines like RANTES (regulated upon activation, normal T-cell expressed and secreted) and MIP-1alpha are elevated in the myocardium of infected animals. The increased expression of chemokines like MIG in the myocardium may contribute to the heavy recruitment of activated T cells, and thus subsequently to the establishment and maintenance of *T.cruzi* induced myocarditis (dos Santos PV *et al*., 2001).

The expression of gamma interferon-induced chemokines such as MIG and RANTES are increased in the inflamed myocardium during acute phase of infection and persist during the chronic phase as well. MIG is known to be involved in the selective recruitment of T helper 1 (Th1) cells (Sallusto F *et al*., 1998). As discussed earlier, there is a strong Th1-immune response in acute phase of *T.cruzi* infection with presence of both CD4 and CD8, and characterized by the production of cytokines like gamma interferon, TNF alpha and IL-12 which are important in the control of parasitic infection (Abrahamsohn IA and Coffman RL, 1996). These cytokines cause the activation of trypanocidal activity of macrophages through NO-dependent mechanism (Chandra M *et al*., 2002; Gutierrez FR *et al*., 2009).

However, MIG and other cytokines mentioned above also persist during chronic phase of *T.cruzi* infection, and could play a damaging and harmful role in cardiomyopathy due to CD. NO may directly control the contractile properties of muscle cells and contribute to decreased cardiac function and myocardial damage. This could further enhance and worsen the chemokine-induced inflammation and tissue damage (Machado FS *et al*., 2000).

This study was the first to demonstrate circulating MIG concentration in CD and idiopathic DCM patients with HF. Plasma MIG levels were found to be significantly raised in CD patients with advanced HF (NHA III-IV) compared to control subjects. Even when compared to DCM patients with HF (NYHA I-IV), MIG concentration in CD patients with HF (NYHA I-IV) was notably higher. As stated earlier, inflammation in CD is more severe and intense in comparison to that in idiopathic DCM. Furthermore, mononuclear cell infiltrate together with large areas of confluent fibrosis are seen throughout the myocardium in CD but not in idiopathic DCM (Rossi MA *et al*., 2003; Nunes VL *et al*., 2006). MIG might be one of the cytokines involved in the inflammatory reactions seen here in patients with cardiomyopathy due to CD and as already shown in experimental studies in animals (Sallusto F *et al*., 1998; dos Santo PV *et al*., 2001). In present study, although MIG failed to show any prognostic potency in CD and idiopathic DCM patients, the significant increase in MIG levels as seen in advanced HF patients with CD encourages the need for further studies to be done in order to investigate and explore the possible use of circulating MIG as a diagnostic and/or prognostic marker in CD.

4.5.   Multivariate analysis – use in diagnosis and prognosis of Chagas' disease

Multivariate analysis was used in this study in order to investigate whether two or more cytokines together could show any efficacy in diagnosing and prognosticating patients with CD. Most of the cytokines investigated in present study were regulated in the same manner. Cytokines such as MIF, SCGF beta, HGF, CTACK, and MIG only showed significant elevation in plasma levels in CD patients with advanced HF (NYHA III-IV). There were no significant changes in concentrations of cytokines in asymptomatic CD group or in CD patients with mild to moderate HF (NYHA I-II). In DCM group as well, plasma concentrations of SCGF beta and HGF were increased markedly only in patients with

advanced HF (NYHA III-IV). Therefore, multivariate analysis was unable to find any combination of two or more cytokines that could help in discriminating CD from idiopathic DCM.

However, multivariate analysis did show some efficacy in prognosticating patients with CD and patients with DCM due to idiopathic causes. In case of CD group, it identified two cytokines by imputing, HGF and IL-12p40, which were able to separate 81.9% of survivors from the deceased (sensitivity/specificity of 92.9%/50.0%). Likewise in DCM group, SDF-1alpha, SCF, and MIG were selected as best parameters through imputing, and were able to separate 77.1% of survivors from the deceased (sensitivity/specificity of 76.5%/77.4%).

Although, multivariate analysis was unable to find a group of cytokines that could together discriminate CD from DCM due to idiopathic causes, it was, however, able to find a number of cytokines that prognosticated a large number of CD patients and idiopathic DCM patients. In future, further studies need to be done in order to identify cytokines that are regulated differently from one another and could possibly have greater efficacy together in diagnosing and prognosticating patients with CD and DCM.

4.6.   Possible role of other cytokines in prognosis of Chagas' disease

There is also a possibility that many other cytokines apart from the ones that we already investigated are regulated in CD and could potentially be used as prognostic markers in the future. Owing to the intensity and severity of inflammation in CD, it is possible that various other cytokines may be involved in the process. The immunological imbalance between pro- and anti-inflammatory cytokines is largely responsible for the myocardial damage seen in CD (Guedes PM *et al*., 2012). Pro-inflammatory cytokines such as interferon gamma, TNF alpha, and IL-6 are already known to be elevated in serum of CD patients (Mocelin AO *et al*., 2005; Marin-Neto JA *et al*., 2007), while the levels of anti-inflammatory cytokines, IL-10 and IL-17, are reduced with increasing severity of HF in CD patients. This decreased production of the cytokines IL-10 and IL-17 together with raised levels of TNF alpha and interferon gamma is correlated with severity of CD (Guedes PM *et al*., 2012). Also, cytokines that promote fibrosis and extracellular matrix remodeling might be regulated in CD as cardiomyopathy due to CD is characterized by diffuse and widespread fibrosis within the myocardium (Rossi MA *et al*., 2003; Nunes VL *et al*., 2006). Therefore, further studies

are required to identify other pro- and anti-inflammatory cytokines and those that induce fibrosis and extracellular matrix remodeling.

4.7.   Conclusion

As mentioned above, HF is characterized by the activation of various inflammatory processes that result in increased levels of inflammatory markers such as IL-6 and CRP (Anker SD and von Haehling S, 2004). Cytokines levels are increased in patients with HF (Torre-Amione G *et al*., 1996), and are associated with the development of ventricular remodeling and progression of HF (Kelly RA and Smith TW, 1997; Li YY *et al*., 2000). Likewise, inflammation mediated by cytokines plays an important role in pathogenesis and progression of CD. However, inflammation in CD is much more severe and has an early, rapid onset as compared to in idiopathic DCM. Inflammatory infiltrate is present throughout the myocardium and there is widespread, diffuse fibrosis (Rossi M *et al*., 2003; Nunes VL *et al*., 2006). This widespread myocardial inflammation can lead to a more severe ventricular remodeling process, causing a rapid and severe onset HF, and ultimately death. The more intense inflammatory activation might be responsible for the poorer prognosis seen in CD when compared to DCM due to idiopathic causes.

Owing to the increased severity of HF in CD, inflammatory pathways and biomarkers are regulated differently compared to in idiopathic DCM (Mocelin AO *et al*., 2005). By further identifying such serum inflammatory cytokines, we might be able to use them in future as tools for early prediction and prognosis of CD. In present study, twenty-one different cytokines were investigated in patients with CD and idiopathic DCM. Although, a number of cytokines such as HGF, MIG, and SCGF beta were found to be significantly elevated in serum of CD patients with advanced HF, none of them showed any potent predictive power for risk in lethality or necessity for heart transplantation. Perhaps some of the cytokines that were studied are only regulated in patients with HF due to specific etiologies. HGF, for example, is known to be a strong and independent predictor of mortality in patients with advanced HF. However, this association is only seen in patients with ischemic HF and not in those with HF from non-ischemic causes like CD (Rychli K *et al*., 2011). It is also possible that various other cytokines apart from the ones that were investigated here are involved in causing severe inflammation and widespread fibrosis seen in CD (Guedes PM *et al*., 2012).

Studies in future should focus more on identifying cytokines that are involved in chronic myocardial and systemic inflammatory activation seen in patients with CD.

Although multivariate analysis was unable to discriminate patients with CD from patients with idiopathic DCM, it did find some cytokines that were together able to separate a large percentage of survivors from the deceased in both CD and DCM groups. However, most of the cytokines in this study were regulated in the same manner, and further studies should be encouraged in the future to identify cytokines that are regulated differently from one another, and could therefore have greater diagnostic and prognostic potency in CD and DCM.

Considering that HF due to CD is associated with poor prognosis and survival (Pereira Nunes Mdo C *et al*., 2010; Barbosa AP *et al*., 2011), there is a need to identify serum biomarkers which could help in predicting the risk of developing DCM in chronically infected, asymptomatic patients. This would help in ensuring that the necessary treatment is provided in time to such patients in order to prevent the onset and progression of HF and thus prevent death. Such biomarkers would also help in prognosis and risk stratification of patients with CD, and in identifying patients who will benefit from heart transplantation.

5. Summary

Chagas' disease (CD), caused by the hemoflagellate protozoan, *Trypanosoma cruzi*, is endemic in most countries of South and Central America, where nearly 10 million people are infected with the parasite and another 25 million are at risk of infection. Cardiac involvement is the most frequent and serious manifestation of chronic CD, and typically leads to abnormalities of conduction system, heart failure (HF), thromboembolism, and sudden death. HF is often a late manifestation of chronic CD and is associated with higher mortality than is HF from other causes. Early identification of patients with CD, therefore, would be desirable as early intervention may help improve prognosis. Inflammatory biomarkers can play a vital role in early diagnosis, as inflammation mediated by cytokines plays an important role in pathogenesis and progression of CD, and may be present even in the absence of HF.

Keeping in view the inflammatory nature of CD, this study investigated the possible role of 21 different inflammatory cytokines as biomarkers for prediction and prognosis of CD. The plasma concentration of each of these cytokines was measured in a group of patients with CD, and then compared with those measured in patients with dilated cardiomyopathy (DCM) from idiopathic causes, and with control subjects. This study was the first to demonstrate cytokines such as monokine induced by interferon gamma (MIG) and stem cell growth factor beta (SCGF beta) in CD and idiopathic DCM patients with HF. Although plasma levels of cytokines like SCGF beta, hepatocyte growth factor (HGF), cutaneous T-cell attracting chemokine (CTACK), and MIG were significantly increased in CD patients with advanced HF, they were unable to show any predictive or prognostic potency in CD. Multivariate analysis was able to prognosticate a large proportion of CD and DCM patients, but it could not discriminate CD from idiopathic DCM. It is possible that some of the cytokines that were investigated here are only regulated in HF due to specific etiologies such as ischemic HF. Also, cytokines other than the ones that were investigated may play a major role in causing severe inflammation and fibrosis seen in CD.

Studies in future should focus on identifying more serum inflammatory biomarkers that could be used as tools for early prediction and prognosis of CD. As CD is associated with poor prognosis, identifying patients at an early stage of the disease may help in providing effective treatment and prevent the development of HF and thus early death.

6. Zusammenfassung

Die Chagas-Krankheit (CD) ist eine von *Trypanosoma cruzi* verursachte Protozoeninfektion, die endemisch in den meisten Ländern Süd-und Mittelamerikas auftritt und 10 Millionen Menschen betrifft sowie weitere 25 Millionen Menschen gefährdet. Ein Herzphänotypist ist die häufigste und schwerste Manifestation der chronischen CD und führt meistens zu Störungen des Reizleitungssystems, zu Herzinsuffizienz (HF), Thromboembolien und plötzlichem Tod. Die HF ist oft eine späte Folge der CD und ist mit einer höheren Sterblichkeit verbunden als eine durch andere Ursachen hervorgerufene HF. Die Früherkennung der CD in Patienten wäre daher wünschenswert, da frühe Intervention zur Prognoseverbesserung führen kann. Entzündliche Biomarker können eine entscheidende Rolle bei der frühen Diagnose spielen, da die von Zytokinen vermittelte Entzündung eine wichtige Funktion in der Pathogenese und Progression von CD spielt und auch in Abwesenheit von einer HF vorhanden sein kann.

Unter Berücksichtigung der entzündlichen Eigenschaften der CD ist in der vorliegenden Arbeit, die mögliche Rolle von 21 verschiedenen inflammatorischen Zytokinen als Biomarker für die Diagnose und Prognose der CD untersucht worden. Die Plasmakonzentration jedes dieser Zytokine wurde in einer Gruppe von Patienten mit CD gemessen und anschließend mit Werten von Patienten mit dilatativerKardiomyopathie (DCM) aus idiopathischen Gründen sowie mit den Werten von Kontrollpersonen verglichen. Die Studie zeigte zum ersten Mal Zytokine wie ,monokine induced by interferon gamma' (MIG) und Stammzellwachstumsfaktor beta (SCGF Beta) in CD- und idiopathischen DCM-Patienten im Zusammenhang mit HF. Obwohl die Plasmaspiegel von Zytokinen wie SCGF beta, Hepatozyten-Wachstumsfaktor (HGF), kutanem T-Zell-anziehendem Chemokin (CTACK) und MIG in CD-Patienten mit fortgeschrittener HF deutlich erhöht waren, konnte ihre diagnostische oder prädiktive Potenz in der CD nicht nachgewiesen werden. Die Multivariate Analyse konnte nicht zwischen CD-Patienten und idiopathischen DCM-Patienten unterscheiden, obwohl der klinische Verlauf für einen großen Anteil der CD- und DCM-Patienten richtig prognostiziert werden konnte. Es ist möglich, dass einige der hier untersuchten Zytokine nur in spezifischen Ätiologien der HF, wie z.B. der Ischämischen HF, reguliert werden. Außerdem können Zytokine, die von uns

nicht untersucht worden sind ebenfalls eine Rolle bei der Entstehung von schweren Entzündungen und Fibrose spielen, die bei CD beobachtet werden.

Deshalb besteht die Notwendigkeit, dass sich weitere Studien mit der Identifizierung entzündlicher Serum-Biomarker, als Hilfsmittel für die frühe Diagnose und Prognose von CD, befassen. Da die chronische Chagas-Kardiomyopathie mit einer schlechten Prognose einhergeht, kann die Früherkennung der Krankheit die Einleitung einer wirksamen Behandlung ermöglichen und die Entwicklung einer HF und somit eines frühen Todes verhindern.

7. List of abbreviations

ACE2	Angiotensin converting enzyme2
ACEI	Angiotensin converting enzyme inhibitor
AIDS	Acquired immunodeficiency syndrome
Apo A1	Apolipoprotein A1
ARB	Angiotensin receptor blocker
Beta NGF	Beta nerve growth factor
BNP	Brain natriuretic peptide
CD	Chagas' disease
CRP	C-reactive protein
CTACK	Cutaneous T-cell attracting chemokine
DALY	Disabiltiy-adjusted life year
DBP	Diastolic blood pressure
DCM	Dilated cardiomyopathy
ECG	Electrocardiogram
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
FGF	Fibroblast growth factor
GRO alpha	Growth related oncogene alpha
HF	Heart failure

HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HR	Heart rate
IFA	Indirect immunofluorescence assay
IFN-alpha2	Interferon-alpha2
IgG	Immunoglobulin G
IL	Interleukin
IL-2R alpha	Interleukin-2 receptor alpha
LIF	Leukemia inhibitory factor
LV	Left ventricular
LVDD	Left ventricular diastolic diameter
LVEDV	Left ventricular end-diastolic volume
LVEF	Left ventricular ejection fraction
LVESV	Left ventricular end-systolic volume
LVSD	Left ventricular systolic diameter
MCP-3	Monocyte chemo-attractant protein-3
M-CSF	Macrophage-colony stimulating factor
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by interferon gamma
MIP-1alpha	Macrophage inflammatory protein-1alpha
NO	Nitric oxide

NYHA	New York heart association
P	p-value, probability
PCR	Polymerase chain reaction
r	Pearson's correlation coefficient
RANTES	Regulated upon activation, normal T-cell expressed and secreted
ROC	Receiver operating characteristic
SBP	Systolic blood pressure
SCF	Stem cell factor
SCGF beta	Stem cell growth factor beta
SDF-1alpha	Stromal derived factor-1alpha
SEM	Standard error of mean
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
vs.	Versus
WHO	World Health Organization

Orders of magnitude:

μ	micro (10^{-6})

m   milli (10^{-3})

p   pico (10^{-12})

Units of measurements:

%   percent

°C   degrees of Celsius

g   gram

L   liter

m   meter

mmHg   millimeter mercury

8. List of figures

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10. Literature

Abrahamsohn, I. A. and R. L. Coffman (1996). "Trypanosoma cruzi: IL-10, TNF, IFN-gamma, and IL-12 regulate innate and acquired immunity to infection." Exp Parasitol**84**(2): 231-44.

Acquatella, H. (2007). "Echocardiography in Chagas heart disease." Circulation**115**(9): 1124-31.

Aguilar V HM, A.-F. F., Racines V J, Paucar C A. (1999). "Epidemiology of Chagas disease in Ecuador. A brief review." Mem Inst Oswaldo Cruz**94** (Suppl 1): 387-93.

Ahrens I, L. G., Peter K. (2010). "New oral anticoagulant drugs in cardiovascular disease." Thromb Haemost.**104**(1): 49-60.

Al-Abed Y, D. D., Aljabari B, Valster A, Messmer D, Ochani M, Tanovic M, Ochani K, Bacher M, Nicoletti F, Metz C, Pavlov VA, Miller EJ, Tracey KJ. (2005). "ISO-1 binding to the tautomerase active site of MIF inhibits its pro-inflammatory activity and increases survival in severe sepsis." J Biol Chem.**280**(44): 36541-4.

Alexander HR, B. K., Block MI, Fraker DL. (1994). "D-factor/leukaemia inhibitory factor: evidence for its role as a mediator in acute and chronic inflammatory disease." Cytokine. **6**(6): 589-96.

Allman E, B. D., Nasir L. (2009). "Depression and coping in heart failure patients: a review of the literature." J Cardiovasc Nurs. **24**(2): 106-17.

Altclas J, S. A., Dictar M, Luna C, Verón MT, De Rissio AM, García MM, Salgueira C, Riarte A. (2005). "Chagas disease in bone marrow transplantation: an approach to preemptive therapy." Bone Marrow Transplant.**36**(2): 123-9.

Andrade SG, R. A., Magalhaes JB, Ferriolli Filho F, Luquetti AO. (1992). "Specific chemotherapy of Chagas disease: a comparison between the response in patients and experimental animals inoculated with the same strains." Trans R Soc Trop Med Hyg.**86**(6): 624-6.

Andrade, Z. A. (1983). "Mechanisms of myocardial damage in Trypanosoma cruzi infection." Ciba Found Symp**99**: 214-33.

Andrade, Z. A. (1999). "Immunopathology of Chagas disease." Mem Inst Oswaldo Cruz**94** (Suppl 1): 71-80.

Anker SD, v. H. S. (2004). "Inflammatory mediators in chronic heart failure: an overview." Heart**90**(4): 464-70.

Aufderheide AC, S. W., Madden M, Streitz J, Buikstra J, Guhl F, Arriaza B, Renier C, Wittmers LE Jr, Fornaciari G, Allison M. (2004). "A 9,000-year record of Chagas' disease." Proc Natl Acad Sci U S A**101**(7): 2034-9.

Barbosa AP, C. N. A., Otaviano AP, Rocha BF, Bestetti RB. (2011). "Comparison of outcome between Chagas cardiomyopathy and idiopathic dilated cardiomyopathy." Arq Bras Cardiol.**97**(6): 517-25.

Bern C, M. S. (2009). "An estimate of the burden of Chagas disease in the United States." Clin Infect Dis**49**(5): e52-4.

Bern C, M. S., Herwaldt BL, Rassi A Jr, Marin-Neto JA, Dantas RO, Maguire JH, Acquatella H, Morillo C, Kirchhoff LV, Gilman RH, Reyes PA, Salvatella R, Moore AC. (2007). "Evaluation and treatment of chagas disease in the United States: a systematic review." JAMA.**298**(18): 2171-81.

Bernstein, R. E. (1984). "Darwin's illness: Chagas' disease resurgens." J R Soc Med**77**(7): 608-9.

Bittencourt AL. (1976). "Congenital Chagas disease." Am J Dis Child. **130**(1): 97-103.

Bocchi EA, F. A. (2001). "The paradox of survival results after heart transplantation for cardiomyopathy caused by Trypanosoma cruzi. First Guidelines Group for Heart Transplantation of the Brazilian Society of Cardiology." Ann Thorac Surg. **71**(6): 1833-8.

Bonney, K. M. and D. M. Engman (2008). "Chagas heart disease pathogenesis: one mechanism or many?" Curr Mol Med**8**(6): 510-8.

Borish L, R. R. (1992). "Physiological studies with human leukocyte inhibitory factor." Immunol Ser. **57**: 373-85.

Braga JC, R. F., Aras R, Costa ND, Bastos C, Silva R, Soares A, Moura Júnior A, Asfora S, Latado AL. (2006). "[Clinical and therapeutics aspects of heart failure due to Chagas disease]." Arq Bras Cardiol. **86**(4): 297-302.

Brandalise NA, A. N., Leonardi LS, Callejas Neto F. (1985). "Carcinoma associated with Chagas' megaesophagus." Rev Col Bras Cir**12**: 196-99.

Britto CC. (2009). "Usefulness of PCR-based assays to assess drug efficacy in Chagas disease chemotherapy: value and limitations." Mem Inst Oswaldo Cruz.**104**(Suppl 1): 122-35.

CA., D. (2000). "Proinflammatory cytokines." Chest.**118**(2): 503-8.

Carod-Artal FJ, V. A., Horan TA, Nunes LG. (2005). "Chagasic cardiomyopathy is independently associated with ischemic stroke in Chagas disease." Stroke**36**(5): 965-70.

Cassatella MA, M. L., Bonora S, Ceska M, Constantin G. (1993). "Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide." J Exp Med. **178**(6): 2207-11.

Centers for Disease Control and Prevention (CDC). (2006). "Chagas disease after organ transplantation--Los Angeles, California, 2006." MMWR Morb Mortal Wkly Rep. **55**(29): 798-800.

"Chagas disease (American trypanosomiasis) fact sheet (revised in June 2010)." Wkly Epidemiol Rec. **85**(34): 334-6.

Chandra M, T. H., Petkova SB, Huang H, Weiss LM, Wittner M, Factor SM, Shtutin V, Jelicks LA, Chan J, Shirani J. (2002). "Significance of inducible nitric oxide synthase in acute myocarditis caused by Trypanosoma cruzi (Tulahuen strain)." Int J Parasitol.**32**(7): 897-905.

Chowdhury IH, A. A., Choudhuri S, Sen A, Hazra A, Pal NK, Bhattacharya B, Bahar B. (2014). "Alteration of serum inflammatory cytokines in active pulmonary tuberculosis following anti-tuberculosis drug therapy." Mol Immunol. **62**(1): 159-68.

Cohn JN, F. R., Sharpe N. (2000). "Cardiac remodeling--concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling." J Am Coll Cardiol. **35**(3): 569-82.

Cohn JN, L. T., Olivari MT, Garberg V, Lura D, Francis GS, Simon AB, Rector T. (1984). "Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure." N Engl J Med**311**(13): 819-23.

Collison A, F. P., Mattes J. (2009). "Emerging role of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) as a key regulator of inflammatory responses." Clin Exp Pharmacol Physiol. **36**(11): 1049-53.

Croxford AL, K. P., Becher B. (2014). "IL-12-and IL-23 in health and disease." Cytokine Growth Factor Rev.

Cui Y, M. P. (2011). "The role of chemokines, cytokines and adhesion molecules in stem cell trafficking and homing." Curr Pharm Des.**17**(30): 3271-9.

Damås JK, E. H., Oie E, Bjerkeli V, Yndestad A, Ueland T, Tonnessen T, Geiran OR, Aass H, Simonsen S, Christensen G, Froland SS, Attramadal H, Gullestad L, Aukrust P. (2000).

"Myocardial expression of CC- and CXC-chemokines and their receptors in human end-stage heart failure." Cardiovasc Res.**47**(4): 778-87.

de Andrade AL, Z. F., de Oliveira RM, Almeida Silva S, Luquetti A, Travassos LR, Almeida IC, de Andrade SS, de Andrade JG, Martelli CM. (1996). "Randomised trial of efficacy of benznidazole in treatment of early Trypanosoma cruzi infection." Lancet. **348**(9039): 1407-13.

Dickstein K, C.-S. A., Filippatos G, McMurray JJ, Ponikowski P, Poole-Wilson PA, Strömberg A, van Veldhuisen DJ, Atar D, Hoes AW, Keren A, Mebazaa A, Nieminen M, Priori SG, Swedberg K; ESC Committee for Practice Guidelines (CPG). (2008). "ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the diagnosis and treatment of acute and chronic heart failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM)." Eur J Heart Fail**10**(10): 933-89.

Diez CN, M. S., Zanuttini JC, Bottasso O, Marcipar I. (2008). "The value of molecular studies for the diagnosis of congenital Chagas disease in northeastern Argentina." Am J Trop Med Hyg. **78**(4): 624-7.

dos Santos PV, R. E., Santiago HC, Torres RA, Marino AP, Paiva CN, Silva AA, Gazzinelli RT, Lannes-Vieira J. (2001). "Prevalence of CD8(+)alpha beta T cells in Trypanosoma cruzi-elicited myocarditis is associated with acquisition of CD62L(Low)LFA-1(High)VLA-4(High) activation phenotype and expression of IFN-gamma-inducible adhesion and chemoattractant molecules." Microbes Infect. **3**(12): 971-84.

Fabbro DL, S. M., Arias ED, Bizai ML, del Barco M, Amicone NA. (2007). "Trypanocide treatment among adults with chronic Chagas disease living in Santa Fe city (Argentina), over a mean follow-up of 21 years: parasitological, serological and clinical evolution." Rev Soc Bras Med Trop.**40**(1): 1-10.

Fiorelli AI, S. N., Honorato R, Bocchi E, Bacal F, Uip D, Strabelli T, Issa V, Amato VA, Fiorelli LR, Oliveira SA. (2005). "Later evolution after cardiac transplantation in Chagas' disease." Transplant Proc.**37**(6): 2793-8.

Flores-Chávez M, F. B., Puente S, Torres P, Rodríguez M, Monedero C, Cruz I, Gárate T, Cañavate C. (2008). "Transfusional chagas disease: parasitological and serological monitoring of an infected recipient and blood donor." Clin Infect Dis**46**(5): e44-7.

Fortini C, T. B., Fucili A, Puppato E, Olivares A, Beltrami AP, Fiorelli V, Bergamin N, Cesselli D, Morelli C, Francolini G, Ferrari R, Beltrami CA. (2011). "Circulating stem cell vary with NYHA stage in heart failure patients." J Cell Mol Med.**15**(8): 1726-36.

Fox N, G. J. (2004). "Biomarkers and surrogates. ." NeuroRx**1**(2): 181.

Franco-Paredes C, V. A., Hidron A, Rodríguez-Morales AJ, Tellez I, Barragán M, Jones D, Náquira CG, Mendez J. (2007). "Chagas disease: an impediment in achieving the Millennium Development Goals in Latin America." BMC Int Health Hum Rights**7**: 7.

Freilij H, A. J. (1995). "Congenital Chagas' disease: diagnostic and clinical aspects." Clin Infect Dis.**21**(3): 551-5.

Freitas HF, C. P., Paes AT, Lima AC, Mansur AJ. (2005). "Risk stratification in a Brazilian hospital-based cohort of 1220 outpatients with heart failure: role of Chagas' heart disease." Int J Cardiol**102**(2): 239-47.

Fu Q, Z. J., Van Eyk JE. (2010). "Comparison of multiplex immunoassay platforms." Clin Chem.**56**(2): 314-8.

Schmunis GA. (2007). "Epidemiology of Chagas disease in non-endemic countries: the role of international migration." Mem Inst Oswaldo Cruz**102**(Suppl 1): 75-85.

Garcia-Alvarez A, S. M., Pinazo MJ, Regueiro-Cueva A, Posada E, Poyatos S, Ortiz-Pérez JT, Heras M, Azqueta M, Gascon J, Sanz G. (2010). "Chagas cardiomyopathy: the potential of diastolic dysfunction and brain natriuretic peptide in the early identification of cardiac damage." PLoS Negl Trop Dis**4**(9).

Garcia RL, M. B., Féres O, Rocha JJ. (2008). "Surgical treatment of Chagas megacolon. Critical analysis of outcome in operative methods." Acta Cir Bras. **23**(Suppl 1): 83-92.

Gascon J, B. C., Pinazo MJ. (2010). "Chagas disease in Spain, the United States and other non-endemic countries." Acta Trop**115**(1-2): 22-7.

Gaspersic R, K. U., Glisovic S, Cör A, Skaleric U. (2010). "Anti-NGF treatment reduces bone resorption in periodontitis." J Dent Res.**89**(5): 515-20.

Gomes YM, L. V., Luquetti AO. (2009). "Diagnosis of Chagas disease: what has been achieved? What remains to be done with regard to diagnosis and follow up studies?" <u>Mem Inst Oswaldo Cruz</u>**104** (Suppl 1): 115-21.

Gonzalez J, M. S., Delage C, Calise D, Maoret JJ, Pradère JP, Klein J, Buffin-Meyer B, Van der Veen B, Charo IF, Heeringa P, Duchene J, Bascands JL, Schanstra JP. (2013). "Dual effect of chemokine CCL7/MCP-3 in the development of renal tubulointerstitial fibrosis." <u>Biochem Biophys Res Commun.</u> **438**(2): 257-63.

Gorlin J, R. S., Robertson G, Stallone F, Hirschler N, Nguyen KA, Gilcher R, Fernandes H, Alvey S, Ajongwen P, Contestable P, Warren H. (2008). "Evaluation of a new Trypanosoma cruzi antibody assay for blood donor screening." <u>Transfusion.</u>**48**(3): 531-40.

Biomarkers Definitions Working Group. (2001). "Biomarkers and surrogate endpoints: preferred definitions and conceptual framework." <u>Clin Pharmacol Ther.</u>**69**(3): 89-95.

Guedes PM, G. F., Silva GK, Dellalibera-Joviliano R, Rodrigues GJ, Bendhack LM, Rassi A Jr, Rassi A, Schmidt A, Maciel BC, Marin Neto JA, Silva JS. (2012). "Deficient regulatory T cell activity and low frequency of IL-17-producing T cells correlate with the extent of cardiomyopathy in human Chagas' disease." <u>PLoS Negl Trop Dis.</u>**6**(4): 1630.

Guerri-Guttenberg RA, G. D., Ambrosio G, Milei J. (2008). "Chagas cardiomyopathy: Europe is not spared!" <u>Eur Heart J.</u> **29**(21): 2587-91.

Gutierrez FR, M. T., Pavanelli WR, Guedes PM, Silva JS. (2009). "The effects of nitric oxide on the immune system during Trypanosoma cruzi infection." <u>Mem Inst Oswaldo Cruz.</u> **104**(Suppl 1): 236-45.

Herbella FA, A. J., Stefani-Nakano S, Artifon EL, Sakai P, Crema E, Andreollo NA, Lopes LR, de Castro Pochini C, Corsi PR, Gagliardi D, Del Grande JC. (2008). "Treatment of achalasia: lessons learned with Chagas' disease." <u>Dis Esophagus.</u> **21**(5): 461-7.

Hiraoka A, S. A., Seki T, Nagasawa T, Ohta N, Shimonishi M, Hagiya M, Shimizu S. (1997). "Cloning, expression, and characterization of a cDNA encoding a novel human growth factor for primitive hematopoietic progenitor cells." <u>Proc Natl Acad Sci U S A.</u> **94**(14): 7577-82.

Hirota H, I. M., Hamaguchi T, Sugiyama S, Murakami E, Kunisada K, Fujio Y, Oshima Y, Nakaoka Y, Yamauchi-Takihara K. (2004). "Circulating interleukin-6 family cytokines and their receptors in patients with congestive heart failure." <u>Heart Vessels.</u>**19**(5): 237-41.

Hobbs FD, K. J., Roalfe AK, Davis RC, Hare R, Davies MK. (2002). "Impact of heart failure and left ventricular systolic dysfunction on quality of life: a cross-sectional study comparing common chronic cardiac and medical disorders and a representative adult population." Eur Heart J**23**(23): 1867-76.

Hohensinner PJ, R. K., Zorn G, Hülsmann M, Berger R, Mörtl D, Richter B, Huber K, Wojta J, Pacher R, Niessner A. (2010). "Macrophage-modulating cytokines predict adverse outcome in heart failure." Thromb Haemost.**103**(2): 435-41.

Hotez PJ. (2014). "Neglected parasitic infections and poverty in the United States." PLoS Negl Trop Dis. **8**(9): e3012.

Hotez PJ, M. D., Fenwick A, Kumaresan J, Sachs SE, Sachs JD, Savioli L. (2007). "Control of neglected tropical diseases." N Engl J Med**357**(10): 1018-27.

Hotez PJ, M. D., Stillwaggon E, Bentwich Z, Kumaresan J. (2006). "Neglected tropical diseases and HIV/AIDS." Lancet**368**(9550): 1865-6.

Ito C, S. H., Ando K, Watanabe S, Yoshiba F, Kishi K, Furuya A, Shitara K, Sugimoto S, Kohno H, Hiraoka A, Hotta T. (2003). "Serum stem cell growth factor for monitoring hematopoietic recovery following stem cell transplantation." Bone Marrow Transplant.**32**(4): 391-8.

Pinto Dias JC. (1995). "Natural history of Chagas' disease." Arq Bras Cardiol**65**(4): 359-66.

Jovic S, S. M., Mörgelin M, Kjellström S, Erjefalt J, Olin AI, Frick IM, Egesten A. (2014). "Expression of MIG/CXCL9 in Cystic Fibrosis and Modulation of Its Activities by Elastase of Pseudomonas aeruginosa." J Innate Immun. .

Juenger J, S. D., Kraemer S, Haunstetter A, Zugck C, Herzog W, Haass M. (2002). "Health related quality of life in patients with congestive heart failure: comparison with other chronic diseases and relation to functional variables." Heart**87**(3): 235-41.

Karimabad MN, H. G. (2014). "Significance of CXCL12 in Type 2 Diabetes Mellitus and Its Associated Complications." Inflammation.

Kasper DL, E. B., Stephen Hauser, Dan Longo, J.Larry Jameson, Anthony S. Fauci. (2005). Harrison's principles of internal medicine. New York, McGraw-Hill Medical Publishing Division.

Kaye DM, V. G., Gruskin SL, Du XJ, Esler MD. (2000). "Reduced myocardial nerve growth factor expression in human and experimental heart failure." Circ Res. **86**(7): 80-4.

Kelly RA, S. T. (1997). "Cytokines and cardiac contractile function." Circulation. **95**(4): 778-81.

Kierszenbaum F, M. L. H., Tanner MK, Sztein MB. (1995). "Trypanosoma cruzi-induced decrease in the level of interferon-gamma receptor expression by resting and activated human blood lymphocytes." Parasite Immunol**17**(4): 207-14.

Koberle, F. (1968). "Chagas' disease and Chagas' syndromes: the pathology of American trypanosomiasis." Adv Parasitol**6**: 63-116.

Kovalchin J, K. J., Genova M, Collins K, Augustyniak M, Masci A, Hittinger T, Kuca B, Edan G, Braudeau C, Rimbert M, Patel U, Mascioli E, Zanelli E. (2010). "Results of a phase I study in patients suffering from secondary-progressive multiple sclerosis demonstrating the safety of the amino acid copolymer PI-2301 and a possible induction of an anti-inflammatory cytokine response." J Neuroimmunol.**225**(1-2): 153-63.

Krumholz HM, C. Y., Vaccarino V, Wang Y, Radford MJ, Bradford WD, Horwitz RI. (2000). "Correlates and impact on outcomes of worsening renal function in patients > or =65 years of age with heart failure." Am J Cardiol**85**(9): 1110-3.

Kusunoki H, T. Y., Otsu R, Rakugi H, Morishita R. (2014). "Anti-inflammatory effects of hepatocyte growth factor on the vicious cycle of macrophages and adipocytes." Hypertens Res. **37**(6): 500-6.

Lamblin N, S. S., Dagorn J, Mouquet F, Jude B, Van Belle E, Bauters C, de Groote P. (2005). "Prognostic significance of circulating levels of angiogenic cytokines in patients with congestive heart failure." Am Heart J.**150**(1): 137-43.

Levin D, S. W., Hoffmann HH, Yarden G, Busetto AG, Manor O, Sharma N, Rice CM, Schreiber G. (2014). "Multifaceted activities of type I interferon are revealed by a receptor antagonist." Sci Signal. **7**(327).

Li YY, F. Y., Kadokami T, McTiernan CF, Draviam R, Watkins SC, Feldman AM. (2000). "Myocardial extracellular matrix remodeling in transgenic mice overexpressing tumor necrosis factor alpha can be modulated by anti-tumor necrosis factor alpha therapy." Proc Natl Acad Sci U S A. **97**(23): 12746-51.

Liu Y, Y. X., Lin QX, Yang M, Shan ZX, Zheng M. (2003). "[Relationship between macrophage migration inhibitory factor and cardiac function in patients with congestive heart failure]." Di Yi Jun Yi Da Xue Xue Bao.**23**(11): 1169-70.

Lloyd Jones-D., Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Roger VL, Thom T, Wasserthiel-Smoller S, Wong ND, Wylie-Rosett J; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. (2010). "Heart disease and stroke statistics--2010 update: a report from the American Heart Association." Circulation. **121**(7): e46-e215.

LM Braz, V. A. N., TS Okay (2008). "Reactivation of Trypanosoma cruzi infection in immunosuppressed patients: contributions for the laboratorial diagnosis standardization." Rev Inst Med Trop Sao Paulo**50**: 2.

Machado FS, M. G., Aliberti JC, Mestriner FL, Cunha FQ, Silva JS. (2000). "Trypanosoma cruzi-infected cardiomyocytes produce chemokines and cytokines that trigger potent nitric oxide-dependent trypanocidal activity." Circulation.**102**(24): 3003-8.

Maltais S, P. L., Ly HQ. (2011). "The bone marrow-cardiac axis: role of endothelial progenitor cells in heart failure." Eur J Cardiothorac Surg.**39**(3): 368-74.

Marin-Neto JA, C.-N. E., Maciel BC, Simões MV. (2007). "Pathogenesis of chronic Chagas heart disease." Circulation. **115**(9): 1109-23.

Marin-Neto JA, M. P., Marcassa C, Gallo Júnior L, Maciel BC, Bellina CR, L'Abbate A. (1992). "Myocardial perfusion abnormalities in chronic Chagas' disease as detected by thallium-201 scintigraphy." Am J Cardiol. **69**(8): 780-4.

Marin Neto JA, S. M., Sarabanda AV. (1999). "Chagas' heart disease." Arq Bras Cardiol**72**(3): 247-80.

Martino TA, L. P., Sole MJ. (1994). "Viral infection and the pathogenesis of dilated cardiomyopathy." Circ Res**74**(2): 182-8.

Martins GA, V. L., Cunha FQ, Silva JS. (1999). "Gamma interferon modulates CD95 (Fas) and CD95 ligand (Fas-L) expression and nitric oxide-induced apoptosis during the acute phase of Trypanosoma cruzi infection: a possible role in immune response control." Infect Immun. **67**(8): 3864-71.

Masson S, L. R., Anand IS, Vago T, Angelici L, Barlera S, Missov ED, Clerico A, Tognoni G, Cohn JN; Val-HeFT Investigators. (2006). "Direct comparison of B-type natriuretic

peptide (BNP) and amino-terminal proBNP in a large population of patients with chronic and symptomatic heart failure: the Valsartan Heart Failure (Val-HeFT) data." Clin Chem**52**(8): 1528-38.

Mathers CD, E. M., Lopez AD. (2007). "Measuring the burden of neglected tropical diseases: the global burden of disease framework." PLoS Negl Trop Dis. **1**(2): e114.

Matsumoto K, N. T. (1996). "Emerging multipotent aspects of hepatocyte growth factor." J Biochem.**119**(4): 591-600.

McMurray, J. J. and M. A. Pfeffer (2005). "Heart failure." Lancet**365**(9474): 1877-89.

Mélik-Parsadaniantz S, R. W. (2008). "Chemokines and neuromodulation." J Neuroimmunol.**198**(1-2): 62-8.

Meyer N, N. S., Rothe T, Siebenhüner A, Akdis CA, Menz G. (2014). "Differential serum protein markers and the clinical severity of asthma." J Asthma Allergy.**7**: 67-75.

Miles MA, F. M., de Arias AR. (2003). "American trypanosomiasis (Chagas' disease) and the role of molecular epidemiology in guiding control strategies." BMJ**326**(7404): 1444-8.

Mio H, K. N., Yokokawa S, Kawai H, Nakagawa S, Takeuchi K, Sekine S, Hiraoka A. (1998). "Isolation and characterization of a cDNA for human mouse, and rat full-length stem cell growth factor, a new member of C-type lectin superfamily." Biochem Biophys Res Commun.**249**(1): 124-30.

Mizuno S, N. T. (2012). "Improvement of sepsis by hepatocyte growth factor, an anti-inflammatory regulator: emerging insights and therapeutic potential." Gastroenterol Res Pract.

Mocelin AO, I. V., Bacal F, Guimarães GV, Cunha E, Bocchi EA. (2005). "The influence of aetiology on inflammatory and neurohumoral activation in patients with severe heart failure: a prospective study comparing Chagas' heart disease and idiopathic dilated cardiomyopathy." Eur J Heart Fail.**7**(5): 869-73.

Moncayo A. (2003). "Chagas disease: current epidemiological trends after the interruption of vectorial and transfusional transmission in the Southern Cone countries." Mem Inst Oswaldo Cruz.**98**(5): 577-91.

Moncayo A, S. A. (2009). "Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy." Mem Inst Oswaldo Cruz**104**(Suppl 1): 17-30.

Mora MC, S. N. O., Marco D, Barrio A, Ciaccio M, Segura MA, Basombrío MA. (2005). "Early diagnosis of congenital Trypanosoma cruzi infection using PCR, hemoculture, and capillary concentration, as compared with delayed serology." J Parasitol**91**(6): 1468-73.

Moreira Mda C, H.-W. S., Wessel N, Moreira Ventura T, Wang Y, Schultheiss HP, Walther T. (2008). "Prognostic value of natriuretic peptides in Chagas' disease: a 3-year follow-up investigation." Cardiology**110**(4): 217-25.

Munger MA, J. B., Amber IJ, Callahan KS, Gilbert EM. (1996). "Circulating concentrations of proinflammatory cytokines in mild or moderate heart failure secondary to ischemic or idiopathic dilated cardiomyopathy." Am J Cardiol.**77**(9): 723-7.

Muñoz J, P. M., Corachan M, Fumadó V, Gascon J. (2007). "Congenital Trypanosoma cruzi infection in a non-endemic area." Trans R Soc Trop Med Hyg**101**(11): 1161-2.

Nakamura Y, M. R., Higaki J, Kida I, Aoki M, Moriguchi A, Yamada K, Hayashi S, Yo Y, Nakano H, Matsumoto K, Nakamura T, Ogihara T. (1996). "Hepatocyte growth factor is a novel member of the endothelium-specific growth factors: additive stimulatory effect of hepatocyte growth factor with basic fibroblast growth factor but not with vascular endothelial growth factor." J Hypertens.**14**(9): 1067-72.

Ndao M, S. T., Caffrey R, Li H, Podust VN, Perichon R, Santamaria C, Ache A, Duncan M, Powell MR, Ward BJ. (2010). "Identification of novel diagnostic serum biomarkers for Chagas' disease in asymptomatic subjects by mass spectrometric profiling." J Clin Microbiol**48**(4): 1139-49.

Nenseter MS, A. P., Ose L, Holven KB. (2014). "Low level of inflammatory marker in hyperhomocysteinemic patients on statin therapy." Scand J Clin Lab Invest.**74**(1): 1-7.

Neubauer, S. (2007). "The failing heart--an engine out of fuel." N Engl J Med**356**(11): 1140-51.

Niessner A, H. P., Rychli K, Neuhold S, Zorn G, Richter B, Hülsmann M, Berger R, Mörtl D, Huber K, Wojta J, Pacher R. (2009). "Prognostic value of apoptosis markers in advanced heart failure patients." Eur Heart J.**30**(7): 789-96.

Nunes VL, R. F., Pimentel Wde S, Fernandes F, Ianni BM, Mady C. (2006). "The role of storage of interstitial myocardial collagen on the overlife rate of patients with idiopathic and Chagasic dilated cardiomyopathy." Arq Bras Cardiol.**87**(6): 757-62.

Opal SM, D. V. (2000). "Anti-inflammatory cytokines." Chest. **117**(4): 1162-72.

Pasic J, L. W., Sullivan MD (2003). "Cytokines in depression and heart failure." Psychosom Med.**65**(2): 181-93.

Patella V, M. I., Arbustini E, Lamparter-Schummert B, Verga L, Adt M, Marone G. (1998). "Stem cell factor in mast cells and increased mast cell density in idiopathic and ischemic cardiomyopathy." Circulation. **97**(10): 971-8.

Pereira Nunes Mdo C, B. M., Ribeiro AL, Amorim Fenelon LM, Rocha MO. (2010). "Predictors of mortality in patients with dilated cardiomyopathy: relevance of chagas disease as an etiological factor." Rev Esp Cardiol.**63**(7): 788-97.

Peters EM, L. C., Spatz K, Daniltchenko M, Joachim R, Gimenez-Rivera A, Hendrix S, Botchkarev VA, Brandner JM, Klapp BF. (2011). "Nerve growth factor partially recovers inflamed skin from stress-induced worsening in allergic inflammation." J Invest Dermatol. **131**(3): 735-43.

Pinto AY, F. A. J., Valente Vda C, Harada GS, Valente SA. (2009). "Urban outbreak of acute Chagas disease in Amazon region of Brazil: four-year follow-up after treatment with benznidazole." Rev Panam Salud Publica.**25**(1): 77-83.

Piper SC, F. J., Kay L, Parker LC, Sabroe I, Sleeman MA, Briend E, Finch DK. (2013). "The role of interleukin-1 and interleukin-18 in pro-inflammatory and anti-viral responses to rhinovirus in primary bronchial epithelial cells." PLoS One.**8**(5): 633-65.

Prencipe G, M. G., Strippoli R, De Pasquale L, Petrini S, Caiello I, Manni L, De Benedetti F, Bracci-Laudiero L. (2014). "Nerve growth factor downregulates inflammatory response in human monocytes through TrkA." J Immunol.**192**(7): 3345-54.

Quiros FR, M. C., Casas JP, Cubillos LA, Silva FA. (2006). "CHARITY: Chagas cardiomyopathy bisoprolol intervention study: a randomized double-blind placebo force-titration controlled study with Bisoprolol in patients with chronic heart failure secondary to Chagas cardiomyopathy [NCT00323973]." Trials.**7**(21).

Rassi A Jr, R. A., Marin-Neto JA. (2009). "Chagas heart disease: pathophysiologic mechanisms, prognostic factors and risk stratification." Mem Inst Oswaldo Cruz**104**(Suppl 1): 152-8.

Rassi A Jr, R. A., Little WC. (2000). "Chagas' heart disease." Clin Cardiol. **23**(12): 883-9.

Rassi A Jr, R. A., Marin-Neto JA (2010). "Chagas disease." THE LANCET**375**(9723): 15.

Rassi A Jr, R. S., Rassi A. (2001). "Sudden death in Chagas' disease." Arq Bras Cardiol**76**(1): 75-96.

Rassi Júnior A, G. R. A., Gabriel Rassi S, Rassi Júnior L, Rassi A. (1995). "Ventricular arrhythmia in Chagas disease. Diagnostic, prognostic, and therapeutic features." Arq Bras Cardiol**65**(4): 377-87.

Reed SG, B. C., Russo DM, Silva JS, Grabstein KH, Morrissey PJ. (1994). "IL-10 mediates susceptibility to Trypanosoma cruzi infection." J Immunol**153**(7): 3135-40.

Reikeras O, B. P., Reseland JE, Lyngstadaas SP. (2014). "Changes in serum cytokines in response to musculoskeletal surgical trauma." BMC Res Notes.**128**(7): 1186.

Robbins SL, K. V., Cotran RS (2003). Robbins basic pathology Philadelphia, Saunders.

Rohde G, M. S., Haas JJ, Kebadze T, Parker H, Laza-Stanca V, Khaitov MR, Kon OM, Stanciu LA, Mallia P, Edwards MR, Johnston SL. (2014). "CXC chemokines and antimicrobial peptides in rhinovirus-induced experimental asthma exacerbations." Clin Exp Allergy.**44**(7): 930-9.

Rosamond W, F. K., Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell C, Roger V, Sorlie P, Steinberger J, Thom T, Wilson M, Hong Y; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. (2008). "Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee." Circulation.**117**(4): 25-146.

Rossi MA. (1990). "Microvascular changes as a cause of chronic cardiomyopathy in Chagas' disease." Am Heart J. **120**(1): 233-6.

Rossi MA, R. S., Bestetti RB. (2003). "Chagas' heart disease: clinical-pathological correlation." Front Biosci.**8**: 94-109.

Russomando G, A. M., Candia N, Franco L, Sánchez Z, de Guillen I. (2005). "Implementation and evaluation of a locally sustainable system of prenatal diagnosis to detect cases of congenital Chagas disease in endemic areas of Paraguay." Rev Soc Bras Med Trop.**38**(Suppl 2): 49-54.

Rychli K, R. B., Hohensinner PJ, Kariem Mahdy A, Neuhold S, Zorn G, Berger R, Mörtl D, Huber K, Pacher R, Wojta J, Niessner A, Hülsmann M. (2011). "Hepatocyte growth factor is a strong predictor of mortality in patients with advanced heart failure." Heart**97**(14): 1158-63.

Sallusto F, L. A., Mackay CR. (1998). "Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses." Immunol Today. **19**(12): 568-74.

San Martín MA, G. A., Rodríguez FJ, Terol I. (2002). "[Dilated cardiomyopathy and autoimmunity: an overview of current knowledge and perspectives]." Rev Esp Cardiol**55**(5): 514-24.

Sartori AM, I. K., Nunes Westphalen EV, Braz LM, Oliveira OC Jr, Gakiya E, Lopes MH, Shikanai-Yasuda MA (2007). "Manifestations of Chagas disease (American trypanosomiasis) in patients with HIV/AIDS." Ann Trop Med Parasitol**101**(1): 31-50.

Scanavacca MI, d. B. F., Maia I, Hachul D, Gizzi J, Lorga A, Rassi A Jr, Filho MM, Mateos JC, D'Avila A, Sosa E; Sociedade Brasileira de Cardiologia. (2002). "Guidelines for the evaluation and treatment of patients with cardiac arrhythmias." Arq Bras Cardiol.**79**(Suppl 5): 1-50.

Scanavacca MI, S. E., Lee JH, Bellotti G, Pileggi F. (1990). "Empiric therapy with amiodarone in patients with chronic Chagas cardiomyopathy and sustained ventricular tachycardia." Arq Bras Cardiol.**54**(6): 367-71.

Silva JS, A. J., Martins GA, Souza MA, Souto JT, Pádua MA. (1998). "The role of IL-12 in experimental Trypanosoma cruzi infection." Braz J Med Biol Res. **31**(1): 111-5.

Silva JS, T. D., Reed SG. (1991). "Regulation of Trypanosoma cruzi infections in vitro and in vivo by transforming growth factor beta (TGF-beta)." J Exp Med**174**(3): 539-45.

Soares MB, P.-D.-C. L., Ribeiro-Dos-Santos R (2001). "The pathogenesis of Chagas' disease: when autoimmune and parasite-specific immune responses meet." An Acad Bras Cienc. **73**(4): 547-59.

Soloski MJ, C. L., Lahey LJ, Wagner CA, Robinson WH, Aucott JN. (2014). "Serum inflammatory mediators as markers of human Lyme disease activity." PLoS One.**9**(4): 932-43.

Song S, C. K., Ryu SW, Kang SW, Choi C. (2011). "TRAIL promotes caspase-dependent pro-inflammatory responses via PKCδ activation by vascular smooth muscle cells." Cell Death Dis. **2**: 223.

Sosa Estani S, S. E., Ruiz AM, Velazquez E, Porcel BM, Yampotis C. (1998). "Efficacy of chemotherapy with benznidazole in children in the indeterminate phase of Chagas' disease." Am J Trop Med Hyg. **59**(4): 526-9.

Srivastava RK, T. G., Barhanpurkar AP, Gupta N, Pote ST, Mishra GC, Wani MR. (2011). "IL-3 attenuates collagen-induced arthritis by modulating the development of Foxp3+ regulatory T cells." J Immunol.**186**(4): 2262-72.

Stein A, P. A., Sison C, Rosen L, Chugh R, Metz C, Bank M, Bloom O. (2013). "Pilot study: elevated circulating levels of the proinflammatory cytokine macrophage migration inhibitory factor in patients with chronic spinal cord injury." Arch Phys Med Rehabil.**94**(8): 1498-507.

Stewart S, J. A., Buchan S, McGuire A, Capewell S, McMurray JJ. (2002). "The current cost of heart failure to the National Health Service in the UK." Eur J Heart Fail. **4**(3): 361-71.

Swedberg K, E. P., Kjekshus J, Wilhelmsen L. (1990). "Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality. CONSENSUS Trial Study Group." Circulation**82**(5): 1730-6.

Nagasawa T. (2014). "CXC chemokine ligand 12 (CXCL12) and its receptor CXCR4." J Mol Med (Berl).**92**(5): 433-9.

Tarhini AA, G. H., Kirkwood JM. (2012). "IFN-α in the treatment of melanoma." J Immunol. **189**(8): 3789-93.

Tarleton, R. L. (2003). "Chagas disease: a role for autoimmunity?" Trends Parasitol**19**(10): 447-51.

Torre-Amione G, K. S., Benedict C, Oral H, Young JB, Mann DL. (1996). "Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: a report from the Studies of Left Ventricular Dysfunction (SOLVD)." J Am Coll Cardiol. **27**(5): 1201-6.

Torrico F, A.-V. C., Suarez E, Rodriguez P, Torrico MC, Dramaix M, Truyens C, Carlier Y. (2004). "Maternal Trypanosoma cruzi infection, pregnancy outcome, morbidity, and mortality of congenitally infected and non-infected newborns in Bolivia." Am J Trop Med Hyg.**70**(2): 201-9.

Vaidian AK, W. L., Tanowitz HB. (2004). "Chagas' disease and AIDS." Kinetoplastid Biol Dis. **3**(1): 2.

Vincent VA, S. S., Murphy GM Jr. (2002). "Proinflammatory effects of M-CSF and A beta in hippocampal organotypic cultures." Neurobiol Aging.**23**(3): 349-62.

Viotti R, V. C., Lococo B, Alvarez MG, Petti M, Bertocchi G, Armenti A. (2009). "Side effects of benznidazole as treatment in chronic Chagas disease: fears and realities." Expert Rev Anti Infect Ther.**7**(2): 157-63.

Viotti R, V. C., Lococo B, Bertocchi G, Petti M, Alvarez MG, Postan M, Armenti A. (2006). "Long-term cardiac outcomes of treating chronic Chagas disease with benznidazole versus no treatment: a nonrandomized trial." Ann Intern Med.**144**(10): 724-34.

Vistnes M, W. A., Nygård S, Sjaastad I, Andersson KB, Husberg C, Christensen G. (2010). "Circulating cytokine levels in mice with heart failure are etiology dependent." J Appl Physiol (1985)**108**(5): 1357-64.

von Haehling S, S. J., Lainscak M, Doehner W, Anker SD. (2009). "Inflammatory biomarkers in heart failure revisited: much more than innocent bystanders." Heart Fail Clin.**5**(4): 549-60.

Wang F, H. D., Tang X, Zhang X. (2014). "Chemokine expression in diverse nonimmediate drug hypersensitivity reactions: focus on thymus activation-regulated chemokine, cutaneous T-cell-attracting chemokine, and interleukin-10." Ann Allergy Asthma Immunol. **113**(2): 204-8.

Wang Y, M. M., Khan A, Heringer-Walther S, Schultheiss HP, Wessel N, Siems WE, Walther T. (2012). "Prognostic Significance of Circulating Levels of Hepatocyte Growth Factor in Patients with Chagas' Disease and Idiopathic Dilated Cardiomyopathy." Cardiology. **121**(4): 240-246.

Wang Y, M. M. C., Heringer-Walther S, Ebermann L, Schultheiss HP, Wessel N, Siems WE, Walther T. (2010). "Plasma ACE2 activity is an independent prognostic marker in Chagas' disease and equally potent as BNP." J Card Fail**16**(2): 157-63.

Won HY, M. H., Ahn JH, Yoo SE, Bae MA, Hong JH, Hwang ES. (2010). "Anti-allergic function and regulatory mechanisms of KR62980 in allergen-induced airway inflammation." Biochem Pharmacol.**79**(6): 888-96.

World Health Organization, "Control of Chagas disease: second report of the WHO expert committee".Geneva (2002).

World Health Organization, "The World Health Report 2006 - working together for health", http: //www.who.int./whr/2006/en/index.html.2006.

Young C, L. P., Chawla A, Glasser L, Forman E. (2007). "Transfusion-acquired Trypanosoma cruzi infection." Transfusion**47**(3): 540-4.

Zhang JM, A. J. (2007). "Cytokines, inflammation, and pain." <u>Int Anesthesiol Clin.</u> **45**(2): 27-37.

Zhu Y, H. Y., Ikeda U, Shimada K. (2000). "Production of hepatocyte growth factor during acute myocardial infarction." <u>Heart.</u> **83**(4): 450-5.

11. List of publications

Journals:

Khan A, Wang Y, Schultheiss HP, Moreira Mda C, Walther T:

Role of monokine induced by interferon gamma (MIG) in discrimination and prognosis of patients with Chagas' disease and idiopathic dilated cardiomyopathy.

Cytokine, **under review. Submitted January 2015.**

Wang Y, **Khan A**, Heringer-Walther S, Schultheiss HP, Moreira Mda C, Walther T:

Prognostic value of circulating levels of stem cell growth factor beta (SCGF beta) in patients with Chagas' disease and idiopathic dilated cardiomyopathy.

Cytokine (2013) 61(3): 728-31

Wang Y, Moreira Mda C, **Khan A**, Heringer-Walther S, Schultheiss HP, Wessel N, Siems WE, Walther T:

Prognostic Significance of Circulating Levels of Hepatocyte Growth Factor in Patients with Chagas' disease and Idiopathic Dilated Cardiomyopathy.

Cardiology (2012) 121(4): 240-246

Wang Y, Moreira Mda C, Heringer-Walther S, **Khan A**, Schultheiss HP, Wessel N, Siems WE, Walther T:

Does the aminopeptidase A (APA) have prognostic and diagnostic value in Chagas disease and other dilated cardiomyopathies?

Journal of cardiovascular pharmacology (2011) 58(4): 374-9

Manuscript in preparation:

Khan A, Wang Y, Heringer-Walther S, Schultheiss HP, Moreira Mda C, Walther T:

Risk stratification with multiple cytokines in patients with Chagas' disease and idiopathic dilated cardiomyopathy.

Poster presentation:

34. Wissenschaftlichen Kongress der Deutschen Hochdruckliga e.V. DHL® - Deutschen Hypertonie Gesellschaft.
09-11 December 2010 in Berlin, Germany.

"Does the aminopeptidase A (APA) have prognostic and diagnostic value in Chagas disease and other dilated cardiomyopathies?"

Wang Y, Moreira Mda C, Heringer-Walther S, **Khan A**, Schultheiss HP, Wessel N, Siems WE, Walther T.

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13. Ehrenwörtliche Erklärung

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