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"Pathogenic mechanisms of tissue damage mediated by *Bartonella henselae* in preclinical models and in patients with advanced heart failure awaiting transplantation"

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List of abbreviations

Bad: Bartonella adhesion
BCVs: Bartonella-containing vacuoles
Bep: Bartonella effector proteins
CSD: Cat Scracht Disease
EPCs: Endothelial Progenitor Cells
HLA: Human Leukocyte Antigens
HIF-1: hypoxia-inducible transcription factor
HPCs: hematopoietic progenitor cells
HTx: Heart transplantation
TAAs: trimeric autotransporter adhesions
T5SS: type V secretion system
VEGF: vascular endothelial growth factor
VEGFR-2: vascular endothelial growth factor receptor

Abstract

Heart transplantation outcome is related to effects of immunological and no immunological factors. The major immunological factors are the HLA, the autoimmunity and the inflammation, while the non-immunological factors include among other the hypertension, diabetes, immunosuppressive therapies, cancer and infections. Infectious complications represent a significant source of morbidity and mortality in heart transplantation recipients. However, few studies have been focused on the incidence and the types of infection in patients awaiting heart transplantation and the impact of pre-transplantation infections on the early post-transplantation period. Often these are opportunistic infections due to virus, bacteria and fungi that can be difficult to identify, to treat, and can be lethal in the immunosuppressed patients. Most post-transplantation infections represent reactivation of latent or quiescent infections that had been contracted prior to transplantation. Recently, several evidences have showed the incidence of *B. henselae* infections in solid organ transplantation recipients. *B. henselae* infections are responsible for a widening spectrum of human diseases, ranging from self-limited to life-threatening and show different course and organ involvement due to the balance between host and pathogen. The role of the host immune response to *B. henselae* is critical in preventing progression to systemic disease. Indeed, in immunocompromised patients, such as solid organ transplanted subjects, *B. henselae* infection results in severe disseminated disease and pathologic vasoproliferation with involvement of different organs mainly due to vasoproliferative lesions. Furthermore, *B. henselae* can persist in primary niche prior to blood-stage infection. *In vitro* and *in vivo* studies have demonstrated that endothelial progenitor cells are able to internalize *B. henselae* and they are eligibile as primary niche. Susceptibility to infections and many other human diseases arises through the complex interaction between environmental and host genetic factors including the HLA molecules. In our study, we have found a *B. henselae* significant antibody positivity rate of 21% in patients awaiting heart transplantation enrolled in our Regional Reference list for organ transplantation. We have demonstrated a significant correlation between the *B. henselae* infection and the presence of HLA-B*35 allele and a significant impairment of number of circulating endothelial progenitor cells in patients with advanced heart failure. In addition, we have also evaluated the tissue damage in a model of immunocompromised mice infected with *B. henselae* by revealing a strong deposition of collagen after *B. henselae* infection and an increase of endothelial progenitor cell number that can suggest an involvement of these cells in the immune response of mice infected but also in the triggering the vasoproliferative process.

Our retrospective observational study, although performed on a small number of patients only in pre-transplantation time suggests that *B. henselae*, together with other emerging bacteria, should be included as a routine analysis in the list of opportunistic infections.

1.0 Background

1.1.0 Cardiovascular disease and HTx

Coronary heart disease, peripheral arterial disease, and endocarditis are the most relevant cardiovascular diseases (World Health Organization, 2011). Several are the causes of cardiovascular diseases but atherosclerosis and hypertension remain the most common (Mathers and Loncar 2006). Despite major risk factors for cardiovascular diseases have been firmly established, still a quarter of people presenting the disease do not show any of the known cardiovascular risk factors (Magnus and Beaglehole, 2001). There is considerable interest to look for novel components affecting cardiovascular health, especially for those that could improve global cardiovascular risk prediction. Based upon the immuno-inflammatory response elicited by infectious disease, the microbiological contribution to atherosclerosis, the pathological basis of the vast majority of cardiovascular diseases, represents a long-standing controversy (Nieto 2002; Rosenfeld and Campbell 2011). An abundance of data suggests that several infectious agents, as *Chlamydophila pneumoniae, Helicobacter pylori,* Cytomegalovirus might contribute to atherosclerotic vascular diseases (Chatzidimitriou et al. 2012). The finding that the bacterium *Bartonella henselae* can infect endothelial progenitor cells (EPCs) (Salvatore et al., 2008) suggests an alternative mechanism for infectious diseases to promote cardiovascular disease. Therefore, this recent evidence, by connecting infective agents, regenerative medicine, and cardiovascular disease, highlights different perspectives about such a blurred scenario.

Heart transplantation (HTx) is the only therapeutical option for patients with end-stage heart failure (Lund et al. 2013) but it is available only to a minority of patients due to the low heart donors availability and the contraindications or risk factors in several patients with heart failure (Hunt and Haddad 2008). Cardiomiopathy has been the leading cause for HTx, followed by coronary artery disease (Stewart et al. 2014). In Italy, patients on awaiting HTx have a waiting list time of about 2.3 years (http://www.trapianti.salute.gov.it/) during which a progressive clinical deterioration and an annual mortality of 8–10% can occur. Indeed, every year 10–15% of patients awaiting HTx are withdrawn from the waiting list because they are no longer eligible candidates. The availability of donor organs remains a limiting factor that results in an increased time on waiting list with higher incidence of mortality (Picascia et al. 2014). This requirement has led to continuous demand in use of mechanical ventricular assist device technologies for the management of patients on waiting for HTx as a bridge to transplantation by improving survival and quality of life of patients with refractory heart failure (Boothroyd et al. 2013).

The identification of patients eligibile for HTx and their inclusion in waiting list is a complex process that requires a multidisciplinary approach to evaluate for each patient the benefit/risk of the transplantation (Crudele et al. 2011, Picascia et al. 2014) The goal of HTx is to increase the length and quality of

life and therefore the perioperative risk, the quality of the transplanted organs as well as the risks of immunosuppressive therapy and the donor-recipient compatibility are all parameters that must be taken into account (Crudele et al. 2011). Other important variables that affect the prognosis and the worsening of clinical conditions are represented by infections that can worsen after transplantation and/or increase the likelihood and degree of complications in the postoperative period.
Furthermore, after the introduction of cyclosporine in the early 1980s, many advances have been made in the diagnosis and treatment of graft dysfunction, in rejection and infection leading to a significant improvement in patient and in graft survival, at least up to 5 years after transplantation (Hunt and Haddad 2008). However, late complications such as cardiac allograft vasculopathy and adverse effects due to prolonged immunosuppressive therapies continue to influence and reduce the long-term survival (Hunt and Haddad 2008). In particular, the early and strong induction immunosuppressive treatment and the long-term maintenance immunosuppressive therapy affect transplantation outcome and the most important side effects are an increased susceptibility to infection and cancer. However, the causes of death were stable in the last years with prevalence of graft failure followed by infections (Lund et al. 2013, Salvadori and Bertoni 2014).

1.1.1 Infection and HTx

To date, only few studies have been focused on the incidence and types of infection in patients with terminal heart failure, although the role of infections in hospital admissions is well known in these patients (Fishman and Issa 2010, Fernández-Ruiz et al. 2014). Although it is recognized that post-transplantation infections affect outcome in HTx recipients and there are many known risk factors for infections especially in the early post-transplantation period, there are no work that describe the epidemiology of infections in patients awaiting HTx, and the impact of pre-transplantation infections on the early post-transplantation period is not well known (Kotton 2007). There are few studies performed on liver transplantation patients with controversial results. Sun (2011) has found no differences in mortality of liver transplantation patients at 90 and 180 days with and without a history of bacterial infection in the year prior to transplantation (Sun et al. 2011). Conversely, Kim (2008) has described colonization or infection prior to liver transplantation as a predictor of death in the first 3 months after transplantation (Kim et al. 2008).
Infectious complications are frequent, but are no longer the leading cause of death. However, late infections are still a threat to long-term graft survival in transplanted patients. Bacterial infections are the most common which usually appear in the first month or after one year following transplantation (Sun et al. 2011). The overall incidence of fungal infections including pulmonary aspergillosis has decreased considerably in recent years (2%-15%), but

associated mortality remains high. Cytomegalovirus is the most important pathogen in HTx, affecting 7%-35% of patients. Although antiviral prophylactic and pre-emptive therapy have reduced the incidence of acute cytomegalovirus disease, the role of this microorganism in the allograft vascular disease, the most common cause of death in these patients, is a cause for concern. Toxoplasmosis, a particularly severe entity in HTx recipients, has decreased with the use of routine prophylaxis in seronegative recipients (Fernández-Ruiz et al. 2014). In addition, driveline and blood stream infections are often a common complication related to ventricular assist device, especially in long-term support with a prevalence ranging from 18 to 59% (Athan et al. 2012, Gordon et al. 2013, Rosenfeldt et al. 2014). In some reports, infections in HTx are associated with significant morbidity and mortality whereas in other studies outcome are not affected.
Several evidences have reported that about the infectious complications, the endocarditis following HTx are unique among solid organ recipients because it is the transplanted organ that becomes infected (Sherman-Weber et al. 2004). The high incidence of endocarditis is related also to catheter and other blood stream infections, donor heart contamination, and suppression of cell-mediated immunity. Unfortunately, the prognosis for post-HTx infective endocarditis is very poor. Moreover, active bacterial infections in the pre-transplantation time are usually considered to be an absolute contraindication to HTx (Macdonald 2008, Durante-Mangoni et al. 2011). However, HTx was resulted as salvage treatment for intractable infective endocarditis (Aymami et al. 2014). Thus infective endocarditis may be the cause for HTx but also a post-transplantation complication.
Although in recent years many advances in the immunosuppressive therapy, in the surgical techniques, in the organ preservation as well as in the overall management of transplantated patients have been made, the infectious complications remain a problem, yet (Crudele et al. 2011). However, the effects of bacterial, fungal, viral and parasitic infections after transplantation depend on the degree of immunosuppression, type of organ transplantated, host factors, as well as post-transplantation period. In the last years, several cases of disseminated *B. henselae* infection have been described in patients underwent to solid organ transplantation including kidney and liver and to a lesser degree heart and lung.

1.1.2 Natural history of *Bartonella* infections

Bartonella spp are facultative intracellular pathogens, gram-negative, aerobic, pleiomorphic bacilli that cause prolonged intraerythrocytic bacteremia and systemic infections in their hosts. To date, more than 30 *Bartonella* spp have been isolated from humans and animals around the world. *Bartonella* spp have a natural cycle of infection common to many pathogens transmitted by vectors which begins with the inoculation of the pathogens in a mammalian reservoir host usually by bloodsucking arthropods (Dehio 2005). After

inoculation, *Bartonella* spp not colonize directly the erythrocytes but remain in a primary niche. Following this step, the *Bartonella* spp are spread into bloodstream and they are able to infect the erythrocytes through several steps ranging from adhesion, invasion and persistence in these cells by enabling a continuous vector transmission (Schülein et al. 2001). This strategy of infection provides to bacteria a sanctuary to defend themselves from the host immune system. During the cycle, the vectors allow to transmit the bacteria from the reservoir host, in which the bacteria cause a chronic intraerythrocytic bacteremia, to new susceptible hosts. There is usually a specific association between the natural host, vectors, and *Bartonella* spp that influences the spectrum of natural or incidental hosts and the geographic distribution of the organisms (Schülein et al. 2001, Boulouis et al. 2005). A wide range of animals serve as reservoirs for *Bartonella* spp but humans are the only known reservoir for *Bartonella bacilliformis* and *Bartonella quintana.* Several species of the genus *Bartonella* cause human infections including *Bartonella henselae*, *Bartonella quintana* and *Bartonella bacilliformis*. *B. henselae* or *B. quintana* provoke pathologies with a limited morbidity while *B. bacilliformis* is unique as a deadly pathogen causing Carrion's disease, which can kill more than 80% of the patients during its acute phase but it is restricted to limited geographic area (Maguiña et al. 2009). The domestic cats are the reservoir of *B. henselae* and the presence of the bacterium in cat fleas has been described. *B. henselae* is transmitted either through the bite, scratch, or lick of an infected kitten or cat. Transmission of *B. henselae* to reservoir occurs via arthropod vectors such as *Ctenocephalides felis*. Furthermore, the multistep strategies of *Bartonella* infection imply that host specificity might be a very complex process in which each step can be modulated at molecular level (Pulliainen and Dehio 2012).

1.1.3 *B. henselae* epidemiology

The different geographic diffusion of the *Bartonella* spp is related to distribution of their hosts or vectors. Indeed, the species with an ubiquitously distribution such as *B. quintana* and *B. henselae* have selected hosts (people and cats) and vectors (body lice and cat fleas) with a worldwide distribution. (Harms and Dehio 2012). *Bartonella* spp. are currently classified as re-emerging pathogens, since various species are increasingly found to elicit human disease, most likely due to changes in human society and medical practice but also thanks to improved diagnostic tools.

Human *Bartonella* infections have been reported from several geographic area, including Europe, but the seroprevalence of antibodies to *B. henselae* have shown a high degree of variation and the antibody prevalence against *B. henselae* in the world may differ in various regions (Mogollon Pasapera et al. 2009; Sun et al. 2010). Epidemiological human studies have greatly increased in the past decade and a high degree of variation in the prevalence of antibodies to *B. henselae* were found in several studies (Vorou et al. 2007; Pandak et al. 2009, Pitassi et al. 2015). Moreover, the seroprevalence of antibodies against

B. henselae among blood donors and otherwise healthy individuals, has been shown to ranged from 0,6 to 61,6% and 0% to 57% respectively (da Costa et al. 2005, Schiellerup et al. 2004, Zarkovic et al. 2007). It was demonstrated a high frequency of serologic evidence of past *B. henselae* infection, in young Italian children, affected by various diseases, apparently free of any clinical features suggesting infection (Mansueto et al. 2012). *B. henselae* infection is frequent in Tuscany and probably underdiagnosed due to the high frequency of atypical onset of the clinical manifestations. In particular, *B. henselae* infection is common among children in central Italy, occurs early in life, in most cases is asymptomatic and resolves spontaneously (Massei et al. 2000 and Massei et al. 2003, Brunetti et al 2013).

1.1.4 Clinical aspects of *B. henselae* infection in humans

B. henselae causes several clinical manifestations in humans but the pathological response and the clinical manifestations of infection are primarily determined by the immune status of the infected human (Resto-Ruiz et al. 2003, Kaiser et al. 2011). These bacteria are rapidly becoming prominent as emerging pathogens causing both acute or chronic infections and vascular proliferative or suppurative manifestations (Mosepele et al. 2012). *B. henselae* infection may cause a variety of human diseases, often symptomless, with different evolution and organ involvement due to the balance between host and pathogenic factors (Mogolon-Pasapera et al. 2009). *B. henselae* shows pathogenic characteristics such as the ability to invade and lyse red cells, causes a persistent bacteremia and induces endothelial cell proliferation. Usually, the response to infection is granulomatous in immunocompetent and vasculoproliferative in immunodeficient individuals. Two manifestations of *B. henselae* infection include Cat Scracht Disease (CSD) and Bacillary angiomatosis-peliosis (Zangwill et al. 2013) (Figure 1). The direct identification of *B. henselae* is difficult due to its slow growing characteristic and, therefore, usually delayed. Unlike molecular and histological methods which require a tissue sample, the serological assays utilize only a small blood sample for a reliable diagnosis. Therefore, diagnosis of *B. henselae* is mainly based on serological methods (IgM, IgG detection) for their ease and fastness (Zangwill et al. 2013).

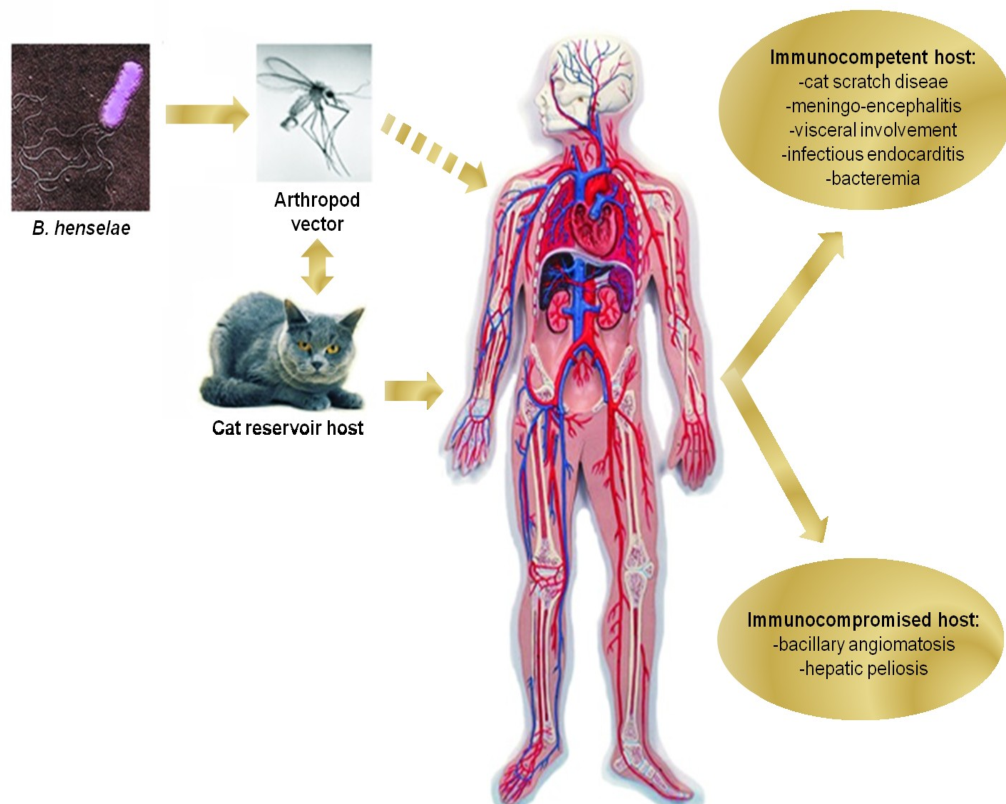

Figure 1. Natural history of *B. henselae* in both immunocompetent and immunocompromised host. *B. henselae* infects a variety of mammals (e.g. cats, dogs and humans) and is transmitted by hematophagous insects. B. henselae causes several clinical manifestation in immunocompromised and immunocompetent hosts.

1.1.5 Cat Scracht Disease

In immunocompetent subjects, these bacteria often cause self-limiting infections usually with fever and regional lymphadenopathy, known as CSD, which can resolve spontaneously without treatment (Zangwill et al. 2013). CSD is a usually self-limiting lymphadenitis with long-lasting swelling of the lymphonodes that drain the primary inoculation site and with an erythematous papule followed by enlargement of lymph nodes at the inoculation site that can persist also for 12-24 months. After few weeks, the lymph nodes regress but in 10% of patients the lymphadenitis may become suppurative. The characteristic histological change in CSD is an inflammatory granulomatous process with central microabscess surrounded by macrophages and rare giant cells. Interestingly, only small number of bacteria have been isolated from lymphonodes supporting the idea that the clinical manifestations are related to immune reaction rather to the pathogen (Prutsky et al. 2013). Additional complications, often in children such as rash, hepatosplenomegaly, lytic bone

lesions, and deep lymphadenitis may also occur. CSD has been reported worldwide and seem to be the most common *B. henselae* infection in people today, occurring with a seasonal incidence especially in children perhaps because they have more likely to have contact with cats. Between 1 and 2% of people with CSD will suffer of neuroretinitis, which includes disk edema and exudates of the macula (Zangwill et al. 2013). In the 1-2% of cases, overall neurological complications can occur including encephalopathy and status epilepticus. Relapsing or systemic infections might be treated with antibiotics. However, a recent meta-analysis study including randomized controlled trials and observational studies demonstrated that antibiotics failed to significantly affect the cure rate or the time required to achieve a cure (Prutsky et al. 2013). It remains unclear whether antibiotic treatment of localized CSD reduces the risk of developing systemic diseases (Angelakis and Rouldt 2014). Diagnosis of *B. henselae* infection can be confirmed by detection of antibodies in the serum of patients, by histopathological examination of lymphonodes or tissue biopsy or by molecular detection of DNA. To date, serodiagnosis by immunofluorescent assay (IFA) is the gold standard for the diagnosis of present and previous infections.

1.1.6 Bacillary angiomatosis

In immunocompromised patients, *B. henselae* results in severe disseminated diseases and pathologic vasoproliferation, known as bacillary angiomatosis and bacillary peliosis, that most commonly involve skin or lymphonodes and liver or spleen, respectively (Mosepele et al. 2012).

Bacillary angiomatosis lesions are the most common manifestations of *B. henselae* infection in immunocompromised patients such as HIV or transplanted patients characterized by cutaneous nodular vascular lesions and compromising of other organs including heart, spleen, kidney, bone and central nervous system (Kaiser et al. 2011, Orsag et al. 2015). Similar lesions are seen also in both bacillary peliosis and splenitis. Bacillary angiomatosis is clinically manifested by the formation of vascular tumors that begin with one or more erythematous papules and they are due to the formation of blood vessels by sprouting of pre-existing vessels for the continued presence of bacteria in the tumor lesions (Pullinaiem and Dehio 2012, Angelakis and Roult 2014). Indeed, the lesions are characterized by proliferating endothelial cells, bacteria and mixed infiltrates of macrophages/monocytes and polymorphonuclear neutrophils. Histologically, these lesions are characterized by the presence of circumscribed proliferation of capillary structures containing epithelial cells, histiocytes and infiltrated neutrophils. These vasoproliferative lesions are indicative of chronic inflammation as consequence of an acute inflammatory reaction triggered by *B.henselae* infected endothelium. In general, an acute inflammatory response induces activation of endothelium that results in the release of proinflammatory chemoattractants and in the establishment of receptor-ligand interactions between endothelium activated and circulating

polymorphonuclear neutrophils (Dehio 2008; Kempf et al. 2005). Epidemiological risk factor analysis showed the cat contact in 84.6% cases of the *B. henselae* infected patients (Angelakis and Rouldt 2014).

1.1.7 Endocarditis and *B. henselae*

B. henselae bacteremia causes endocarditis, primarily in people with existing heart valve abnormalities. Indeed, *B. henselae* has been isolated from native aortic valve tissue of individuals affected by infective endocarditis as agent of blood culture-negative endocarditis (Hoffman et al. 2007, Chomel et al. 2009, Khalighi et al. 2014). *B. henselae* causes a typical endocarditis diagnosed using the Duke criteria showing vegetations detected by echocardiography (Fournier et al. 2010). A recent study and several clinical trials reported that 24% cases of blood culture-negative endocarditis were caused by *B. henselae* (Houpikian and Raoult 2005, Dreier et al. 2008, Tsuneoka et al. 2010, Lamas Cda et al. 2013). The diagnosis of infective endocarditis can be difficult for the atypical presentation and negative blood cultures. Patients show to have pre-existing heart valve diseases that promote the development of infective endocarditis and do not have a history of CSD. *B. henselae* causes significant destruction of the valves characherized by mononuclear cell inflammation, extensive fibrosis, large calcification leading to valvular surgery (Houpikian and Raoult 2005). In addition, *B. henselae* in patients with valvular diseases can induce various clinical presentations such as bacteraemia, hepatic or splenic diseases, encephalitis, cardiopathy and myocarditis (Gouriet et al. 2007). All these studies have emphasized as several heart diseases can be caused from uncultured bacteria such as *B. henselae* and these infections are very often undiagnosed. Endocarditis, myocarditis, dilated cardiomiopathy may indicate the bacterial origin of the diseases and therefore the detection of significant titres of specific antibodies allows to identify the origin of the disease (Million et al. 2010). At this regard, DNA of *B. henselae* was detected in aortic valves of patients undergoing to HTx suggesting that among patients with cardiac disease, infections caused by *B. henselae* should be tested (Nilsson et al. 2005, Maczka et al. 2011). A recent study have demonstrated the need of HTx as final treatment in patients with infective endocarditis with extensive perivalvular lesions and end-stage heart failure (Aymami et al. 2014).

1.1.8 *B. henselae* infection in transplanted patients

In the last years, several cases of disseminated *B. henselae* infection with manifestation of bacillary angiomatosis and peliosis have been described in patients underwent to solid organ transplantation including kidney and liver and to a lesser degree heart and lung. (Juskevicius et al. 2004, Psarros et al. 2012). This distribution of bartonellosis is representative of the frequency of a particular transplanted population rather than the risk of *B. henselae*-mediated

disease in a particular type of transplantation. Furthermore, only 28% of the cases of bartonellosis were aged <18 years of age contrarily to immunocompetent patients in whom *B. henselae* infection occurs mostly in children and adolescents and rarely in older persons (Zangwill et al. 2013). The transplanted patients showed disseminated infection differently to *B. henselae* infection in immunocompetent patients in which usually the only clinical manifestation is the CSD. The clinical features of *B. henselae* infection in transplanted patients are variable and include fever and lymphadenopathy, pulmunary nodules, ocular and skin lesions, osteomyelitis, focal access and hepatosplenic disease (Rheault et al. 2007). The biopsies of lesions showed pathological findings similar to those observed in immunocompetent hosts such as granuloma and suppurative lesions but also vasoproliferative lesions which are found in lymphonodes, liver, skin and bone marrow. In addition, the immunocompromised patients are more likely to develop *B. henselae* bacteremia or other systemic manifestations that can occur at followed to spreading of bacilli in the liver, spleen, and cardiac valve triggering the vasoproliferative lesion as bacillary angiomatosis/peliosis (Rostad et al. 2012, Moulin et al. 2012, Poudel et al. 2014). Interestingly, in immunocompetent subjects *B. henselae* causes a long-lasting bacteremia while in immunodeficients, an alteration of innate and adaptive immunity promotes systemic manifestations due to the difficulty of limiting the infection. The major risk factor for *B. henselae* infection is the contact with cats. Patients with disseminated bartonellosis developed disease at different time of transplantation. In particular, patients developing earlier disease were more at risk of dissemination due to greater immunosuppression. Indeed, the liver transplanted patients have showed a disease earlier post-transplantation because these patients are underwent to a greater degree of immunosuppression (Psarros et al. 2012). Immunosuppressive therapy at which transplanted patients are underwent is a risk factor for bacillary angiomatosis and peliosis due to *B. henselae*. However, there are no clear relationship between the development of disseminated bartonellosis and a particular immunosuppressive therapy.

Several studies have shown that *B. henselae* tend to cause vasoproliferative lesions rather granuloma in HIV patients suggesting that T-cell response of host can play a pivotal role in the control of *B. henselae* infection. Indeed, most of immunosuppressive drugs used in solid organ transplantation target the cellular-mediated immune response, specifically T cells including anti-thymocyte globulin, anti-CD3 antibodies and anti-interleukin-2 receptor that are utilized in the induction phase of immunosuppression. In addition drugs used during the maintenance phase such as cyclosporine or tacrolimus affect the activation, the proliferation and the differentiation directly or indirectly of T cells (Kuypers et al. 2010).

1.2.0 Strategies of *B. henselae* infection: invasion and intracellular colonization of erythrocytes

B. henselae has been demonstrated to establish chronic intraerythrocitic bacteremia in human (Minnick et al. 2009) (Figure 2). However, *B. henselae* not causes an immediate infection of red blood cells but can persist in a primary niche prior to blood-stage infection (Schülein et al. 2001). Moreover, the prolonged persistence intraerythrocytic of *B. henselae* is a essential aspect of its pathogenicity (Dehio 2001). The erythrocytes cannot to present bacterial antigens at immune system due to the lack of major histocompatibilty on their surface and in these cells the bacteria replicate. Unlike of nucleated cells, during the bacterial entry, a small extracellular factor deformin induces indentations of the erythrocytic membrane in which bacteria might localize prior to their internalization. *B. henselae* replicates in the endocytic vacuoles and persists for the lifetime of the erythrocytes which increase the likelihood for its spread through blood-feeding insects.

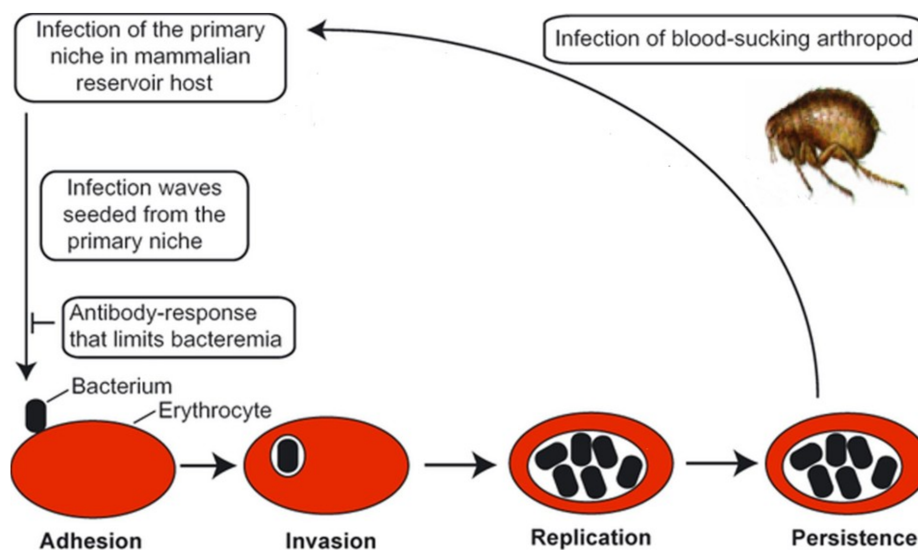

Figure 2: Steps of blood-stage infection by *Bartonella* spp. in their mammalian host. The infection results in colonization of primary niche followed by binding, invasion, replication and persistence in erythrocytes. The coincidental re-infection of primary niche and the efficient transmission to other hosts mediated by ectoparasites occurs.

1.2.1 Cellular characteristics of primary niche

Although several studies have highlighted the main pathogenic mechanisms of *B. henselae* infection, to date it is still unclear what is the primary niche of the bacterium *in vivo* (Schülein et al. 2001, Dehio 2008, Okujava et al. 2014). Several evidence *in vivo* and *in vitro* have reported that

endothelial cells due to a strong interaction and proximity to the circulation can represent an important target of *B. henselae* and are eligible to be part of the primary niche (Dehio 2005). However, other cells as migratory cells may play a crucial role in the establishment of *B. henselae* infection due to the bacterial need to be transported in the host. The involvement of lymph nodes during *B. henselae* infection suggests that lymphocytes or mononuclear phagocytes could be the vehicles of *B. henselae* transport by promoting the migration of pathogen in the tissue and vasculature areas (Dehio 2005). In particular, three loci have been proposed as the primary sites of infection: extracellular matrix, bone-marrow stem cells and mature endothelial cells.
Other studies have proposed hematopoietic progenitor cells (HPCs) as primary niche suggesting that *B. henselae* could infect erythrocyte progenitors and then enter in the bloodstream coincident with their maturation (Mandle et al. 2005). However, it seem evident that the infection of primary cells *in vivo* could contribute to re-infection of the bloodstream and to dissemination of the pathogen. Several *in vitro* studies showed that *B. henselae* can invade endothelial cells, macrophages and monocytes including microglial cells (Musso et al. 2001). On this basis, the ability to invade endothelial cells could strongly alter the maintenance of vascular homeostasis and determine the pathogenic mechanisms of *B. henselae*.

1.2.2 Adhesion and invasion of endothelial and nucleated cells

The interaction of *B. henselae* with endothelial and other nucleated cells occurs by several mechanisms including cellular invasion, activation of NFkB and hypoxia-inducible transcription factor (HIF-1) signaling. The first step is the adherence of bacterium to the extracellular matrix and to the host cell membrane through components of surface structures of *B. henselae* identified as trimeric autotransporter adhesions (TAAs). These non-fimbrial outer membrane adhesion proteins belong to the type V secretion system (T5SS) (Pullinaiem and Dehio 2012). The *Bartonella adhesion A* (BadA) belongs to the TAAs family and it binds some components of extracellular matrix of host cells including collagen, laminin and β1-integrin. The uptake of *B. henselae* in endothelial and other nucleated cells is related to actin-enriched cell membrane protrusions of the host cell (Figure 3). The internalization in endhotelial cells occurs through two different processes enabling either the uptake of single bacteria by a zipper-like pathway and the aggregation of bacteria in large structures defined as invasoma. Single bacteria are internalized in *Bartonella*-containing vacuoles (BCVs) localized in a perinuclear area and that fail to acidify and to fuse with lysosomes due to the lack of lysosomal markers (Kyme et al. 2005). The BCVs-mediated endocytosis is blocked after injection of *Bartonella effector proteins* (BepA-BepG) in the host cells (Truttmann et al. 2011, Eicher and Dehio 2012). Invasoma formation is a multipstep process that involves bacterial adherence, effector translocation, aggregation and internalization by an F-actin- and integrinβ1-dependent mechanism (Truttmann

et al. 2011). Noteworthy, the invasome formation is a slower process than the BCVs development.

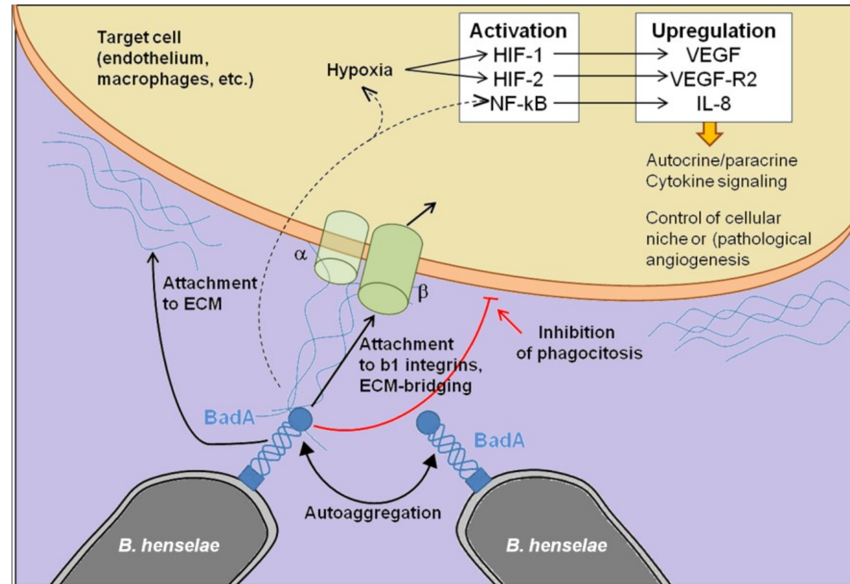

Figure 3. Adhesion of *B. henselae* on target cell. BadA, a trimeric autotransporter adhesion mediates the adhesion on target cells and a direct or indirect relationship between the attachement to β1-integrin and the HIF or NF-κB was supposed.

1.2.3 Virulence factors of *B. henselae*

Virulence factors of *B. henselae* involved in the infection, replication and persistence in host cells have been identified including the Type IV Secretion System (TFSS), LPS, GROEL, and BadA (Vayssier-Taussat et al. 2010, Siamer and Dehio 2015). The TAAs are virulence factors of Gram-negative bacteria that bind host proteins on cell surface (Harms and Dehio 2012) (Figure 4).

BadA

BadA is a trimeric autotrasporter of *B. henselae* involved in the adhesion to endhotelial cells by targeting β1-integrin and by binding to extracellular matrix (Figure 3). BadA promotes a proangiogenic transcriptional program in host cells. The activation of both NFkB and HIF-1 was demonstrated to be dependent on BadA. Kempf (2005) has reported that NFkb and HIF-1 activation are induced by the interaction between β1-integrin and extracellular matrix proteins by leading to the expression of proangiogenic genes such as IL-8 by NFkB pathway (Kempf et al. 2005). Thus, the interaction with BadA would to be involved in the activation of pro-angiogenic program by activating

HIF-1. BadA was demonstrated to protect *B. henselae* from phagocytosis macrophages-mediated (Riess et al. 2004).

VirB-like T4SS and Beps

T4SS are complex molecular machineries that mediate the conjugation process and traslocation of effector proteins in target cells (Dehio 2008).

The VIRB/D4 T4SS of *B. henselae* translocates Beps in the host cells subverting their functions to support the bacteria. The Beps complex shows a modular domain with the N-terminal FIC (filamentation induced by cyclic AMP) that mediates the interaction with the host and a C-terminal composed by a Bep intracellular delivery (BID) domain. Seven Beps are known. The BID domain can trigger a strong antiapoptotic and proangiogenic response (Scheidegger et al. 2011). Some Beps such as BepG and BepC are involved in the invasome pathway by inhibiting the endocytic uptake of *B. henselae* and by promoting the aggregation of bacteria (Rhomberg et al. 2009). Schmid (2006) has found that BID domain display an antiapoptotic activity and Beps participate to proinflammatory activation of endothelial cells by an increase of NFkB activation, ICAM-1 expression and IL-8 secretion. Noteworthy, BadA antibodies are detected in the sera of patients with *B. henselae* infection (Schmid et al. 2006).

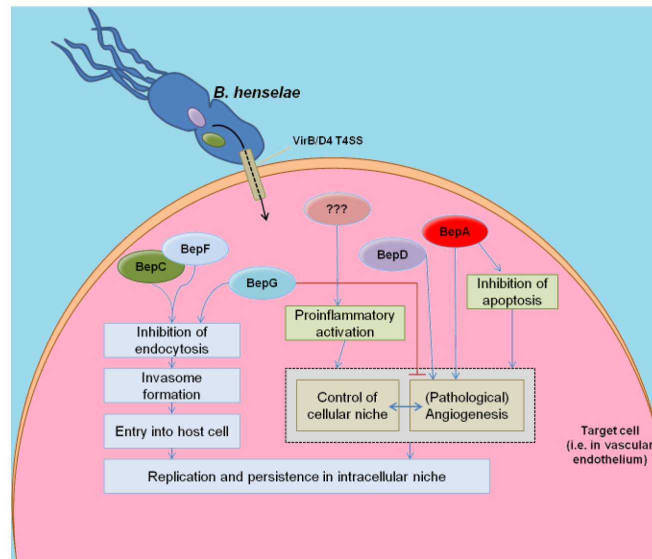

Figure 4. VirB/D4 T4SS and Beps complex. VirB/D4 T4SS contributes to *B. henselae* virulence by translocating Beps in host cells subverting cellular function.

1.2.4 *B. henselae* and mechanism of angiogenesis

The colonization of vascular endothelium and the persistence in endothelial cells are crucial steps for the pathological angiogenesis, the

activation of pro-inflammatory processes and the vasculoproliferative disorders that are triggered from *B. henselae*. In close proximity of proliferating endothelium, bacterial aggregates and dispersed bacteria can be found and the lesions are infiltrated by phagocytes such as macrophages and neutrophils (Pullinaiem and Dehio 2012). To date, it is supposed that *B. henselae* may cause vasculoproliferative disorders through several different mechanisms including NFκB-dependent proinflammatory activation, direct proliferation of endothelial cells, inhibition of endothelial cell apoptosis, and stimulation of angiogenesis through growth factors produced from peripheral cells (Kaiser et al. 2011).
The infiltration of neutrophils in the vasoproliferative lesions after *B. henselae* infection indicates a proinflammatory cascade NF-kB-mediated. Indeed, endothelial cells infected with *B. henselae* have shown an up-regulation of adhesion molecules such as E-selectin, ICAM-1 and the release of chemokines including IL-8 and activation of NFκB (Fuhrmann et al. 2001). Even if the role of the host inflammatory response to *B. henselae* pathogenesis is not well understand, several evidence seem to suggest that the bacteria can use this mechanism to attract key regulators of angiogenesis such as macrophages to the sites of infection, which secrete pro-angiogenic molecules and growth factors (Fuhrmann et al. 2001) (Figure 5).
The proliferation and migration of endothelial cells are crucial steps of the angiogenesis process stimulated by *B. henselae* and depend on a synergistic effect of a direct mitogenic and antiapoptotic stimulation of endothelial cells as well as the autocrine and paracrine cytokine secretion (Resto-Ruiz et al. 2002). The angiogenic stimulation of *B. henselae* occurs through at least two apparently distinct pathways such as secretion of factor stimulating Ca_2 signaling (McCord et al. 2007) and activation of proinflammatory pathways involving NF-kB (Kempf et al. 2005). Furthermore, the angiogenic reprogramming of host cells infected with *B. henselae* seem to be regulated by the activation of HIF-1, a transcriptional factor that stimulates angiogenesis and secretion of vascular endothelial growth factor (VEGF). The secretion of HIF-1 and VEGF has been demonstrated in patients with bacillary angiomatosis and *in vitro* system (Kempf et al. 2005, Riess et al. 2004). There are still many controversies regarding endothelial proliferation stimulated by *B. henselae* because some studies support that direct contact with the pathogen is necessary for proliferation while other evidences underline that endothelial cells can also proliferate separated from bacteria. In additions, it was found that *B. henselae* increases survival of endothelial cells through inhibition of apoptosis mediated by caspases 3 and 8 (Kirby and Nekorchuk 2002) (Figure 5).
Although, a strong transcriptional reprogramming of endothelial cells after *B. henselae* occurs, these cells do not secrete significant amount of VEGF (Dehio 2005; Kempf et al. 2005). Therefore, VEGF detected in lesions angiomatosis perhaps can be released by other cells such as macrophages supporting the hypothesis of a paracrine effect on the endothelial cell proliferation (Resto-

Ruiz et al. 2002). Indeed, endothelial cells incubated with supernatants of *B. henselae*-infected macrophages have showed an increased rate of proliferation through both release of VEGF and pro-angiogenic factors including IL-1β or IL-8 (Resto-Ruiz et al. 2002, McCord et al. 2005). However, massive secretion of IL-8, a pro-angiogenic cytokine was detected in endothelial cells after *B. henselae*-infection, by acting through an autocrine mechanism (McCord et al. 2006). However, this paracrine stimulation can trigger the secretion of various proangiogenic and antiapoptotic factors, especially IL-8 and monocyte chemoattractant protein 1 in endothelial cells, thus promoting the migration of cells and the angiogenic process (McCord et al. 2005) (Figure 5).

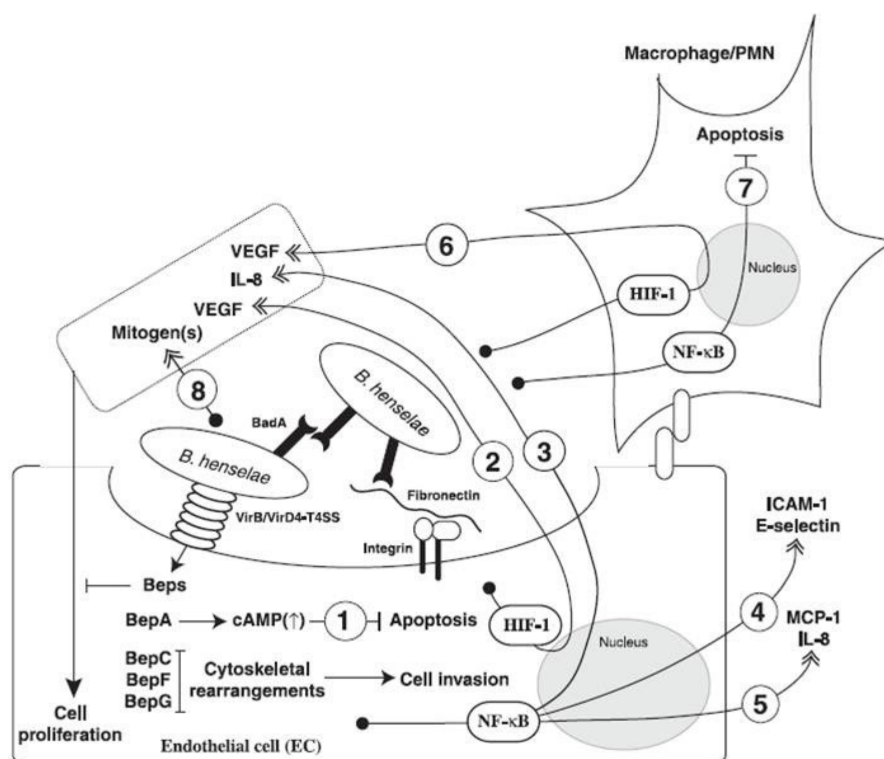

Figure 5. Representation of vascular tumorigenesis due to *B. henselae*. 1) Inhibition of endothelial cells apoptosis. 2-3) Secretion of angiogenic factors from endothelial cells acting on endothelial cells by autocrine manner. 4-5) Induction of adhesion and secretion of chemoattractant molecules. 6) VEGF secretion from macrophages. 7) inhibition of apoptosis. 8) Release of endothelial cell mitogens (modified by Pullianiem et al 2012).

1.2.5 Mechanisms of immune evasion of *B. henselae*

The intracellular persistence of *B. henselae* in host erythrocytes depends on the efficient ability to subvert and to evade the innate and adaptive host immune responses. After the entry of *B. henselae* in the host, the pattern

recognition receptors on the professional phagocytes play a crucial role in the recognition of pathogen. Lipopolysaccharide is an essential outer membrane component in Gram-negative bacteria that with the lipid A portion recognized by a subgroup of pattern recognition receptors, the Toll-like receptors, triggers the secretion of pro-inflammatory cytokines and recruitment of inflammatory cells (Miller et al. 2005). The lipopolysaccharide of *B. henselae* is composed of a unique combination of Lipid A and an uncommon hydroxylated long-chain fatty acid with an unusual structure that might explain the weak Toll-like receptors activation and its less toxicity that promotes the persistent infection and the evasion of immune system (Zähringer et al. 2004). In addition, *B. henselae* resides in membrane-bound intracellular compartments of macrophages and endothelial cells, rather than in the cytoplasm. However, BCVs have showed a delayed development or complete lack of typical endocytic markers allowing to the pathogen of avoid lysosomal fusion and acidification of vacuoles (Kyme et al. 2005). *B. henselae* have the ability to escape from professional phagocyte recognition likely by inhibiting the production of reactive oxygen species, that represents the most important antimicrobial effector mechanisms of these cells.
Furthermore, it was found that *B. henselae* can avoid to elicit the host immune response also through an immunomodulation mechanism. Several studies *in vitro* have demonstrated the involvement of Th1 response in *B. henselae* infection showing an increase of cytokine IFNγ, IL-12 and IL-8. In particular, in immunocompetent subjects, acute *B. henselae* infection causes an upregulation of pro-inflammatory such as IL-2 and IL-6 and anti-inflammatory cytokines including IL-10 (Kabeya et al. 2009). Upregulation of IL-2 can induce a T-cell response in immunocompetent subjects by helping to explain the increased susceptibility of immunocompromised individuals to *B. henselae* infection. Also IL-6 is a marker of inflammation and it is crucial in the induction of an effective immune response. Moreover, IL-10 is a potent anti-inflammatory mediator that controls the cellular immune response to *B. henselae* and its levels correlate strongly with IL-6 levels by suggesting that IL-10 might control the cytokine cascade, perhaps as a protective mechanism (Papadopoulos et al. 2001, Vermi et al. 2006). This cytokine acts as immunomodulator, being able to suppress the function of several immune cells including T helper, monocytes, macrophages influencing both innate and adaptive immune response. It was proposed that the level of IL-10 may pose a balance between a condition favorable both to the host, by preventing tissue damage and to bacteria by attenuating the immune response.

1.3.0 Primary niches other than endothelial cells

Although several studies have highlighted the main pathogenic mechanisms of *B. henselae* infection (Schülein et al 2001, Dehio 2008, Okujava et al. 2014), to date, it is still unclear what is the primary niche of this bacterium *in vivo*.

Due to the demonstrated *in vivo* intraerythrocitic presence of *B. henselae,* the HPCs have been assumed as potential niche and cause of persistent and recurrent intraerythrocitic bacteremia (Eicher et al. 2012). Mandle (2005) has found that *B. henselae* not infects human erythrocytes *in vitro* likely due to the lack β1-integrin required for bacterium binding. Conversely, the infection of CD34$^+$ HPCs with *B. henselae* results in the presence of the pathogen in erytroid cells associated with the persistence and the replication of *B. henselae* in host progenitors cells during differentiation of these cells. The proliferation of HPC infected has been lower respect to cells not infected and HPCs infected have shown an increased secretion of IL-8 that promotes the mobilization of HPCs and thus the propagation and systemic diffusion of pathogen from the bone marrow. Noteworthy, the infection of HPCs results in the diffusion of bacterium through differentiated cells. Moreover, the HPC mobilized can promote the *B. henselae* infection of other organs such as endhotelium and they can be a vehicle to carry *B. henselae* in the sites where vasoproliferative disorders occur. Interestingly, HPCs were resistant to *Listeria monocytogenes, Salmonella enteritica* and *Yersinia enterocolitica* infection *in vitro* (Mandle et al. 2005).
Salvatore (2008) has demonstrated for the first time that *B. henselae* can be adhere to and invade human EPCs *in vivo* and *in vitro* (Salvatore et al. 2008).

1.3.1 Endothelial progenitor cells

EPCs were derived from bone marrow and incorporated into foci of physiological or pathological neovascularization. (Shi et al. 1998; Asahara et al. 1999). These cells have characteristics similar to hematopoietic stem cells due to the common progenitor, the hemangioblast and they are able to differentiate *ex vivo* in cells with endothelial phenotype. Most of the EPCs reside in the bone marrow in close association with the hematopoietic stem and stromal cells, that provide the optimal microenvironment for hematopoiesis. Many studies have shown that heterologous, homologous or autologous EPCs are incorporated in the sites of angiogenesis in animal models of ischemia (Asahara et al 1999). Shi (1998) has identified the presence of EPCs derived from bone marrow in the adult peripheral blood, by demonstrating the presence of a subpopulation able to move through the blood stream in all vascular districts (Shi et al. 1998). Originally, the postnatal neovascularization was promoted by a mechanism of angiogenesis due to *in situ* proliferation and migration of existing endothelial cells. However, the finding that EPCs can home to sites of neovascularization and differentiate into endothelial cells *in situ* is consistent with the vasculogenesis process, a critical paradigm well described for the embryonic neovascularization but also proposed for the adult organism (Rafii et al. 2003, Napoli et al. 2007). Therefore, these studies have revolutionized the idea that postnatal tissue revascularization was achieved by neighbouring endothelial replication and that the differentiation of hemangioblast in vascular endothelial cells occurs only during the embryonic

stage (Napoli et al. 2007, Urbich et al. 2004). EPCs are immature cells that can contribute to the postnatal neovasculogenesis (Rafii et al. 2003, Napoli et al. 2007). Numerous studies have described their stem cell features, their pattern of surface proteins including CD133, VEGFR-2, CD34 and their involvement in physiological as well as pathological neo-vascularisation (Rafii et al. 2002). EPCs are recruited from the bone marrow to sites of hypoxia through a mechanism mediated by HIF and VEGF where can differentiate into endothelial cells and promote the formation of new vessels (Brixius et al. 2006).

1.3.1 EPCs and cardiovascular regeneration

EPCs represent the promoters of vascular repair and a protective factor against cardiovascular disease, acting both at a very early stage of the atherogenesis process and in the final stage of clinical manifestations (Napoli et al. 2011). The negative correlation between cardiovascular risk and the number of circulating EPCs is reinforced by the finding that exposure to certain drugs that favorably affect the cardiovascular risk results in a positive modulation of the number and function of EPCs (Dotsenko 2010, Vasa et al. 2001, Dimmeler et al. 2001). Indeed, after endothelial dysfunction the EPCs are incorporated in the area of vascular damage by supplying endothelial cells for new blood vessels growth and by stimulating the secretion of growth factors. In normal conditions, the endothelium produces and secretes substances that modulate vascular tone and protect the endothelium (Napoli et al. 2011). The loss of structural and functional endothelium triggers complex events that lead to atherosclerosis and cardiovascular diseases, such as myocardial infarction, heart failure and peripheral artery disease. Several studies have focused on the EPC number and their involvement in several human pathologies. In particular, the crucial role of EPCs in neovascularization opens new perspectives in the clinical setting, which include either the revascularization after ischemia or *viceversa* the inhibition of vasculogenesis in oncology (Napoli et al. 2011). The number of EPCs has been reported to negatively correlate with several risk factors for cardiovascular disease such as hypertension and diabetes mellitus (Umemura and Higashi 2008). Levels of EPCs are inversely correlated to progression of coronary artery disease (Liguori et al. 2010). Indeed, the number of EPCs is known to decrease with increasing age associate with a telomere length (Napoli et al. 2007). Changes in EPC number have been also investigated in metabolic disorders (Fadini et al. 2009), neoplastic pathologies (Naik et al. 2008), chronic kidney disease (Jie et al. 2010) and chronic obstructive pulmonary disease (Janssen et al. 2014). Furthermore, increased levels of EPCs have also been associated with other inflammatory conditions such as periodontal infections, rheumatoid arthritis or in situations of acute tissue damage (Napoli et al. 2011).

1.3.2 Identification and characterization of EPCs

Asahara (1997) has described the EPCs after isolation of $CD34^+$ cells from human peripheral blood using magnetic microbeads (Asahara et al. 1997). These cells after plating on fibronectin-coated surfaces have shown endothelial characteristics and positivity for both the binding to lectin and the incorporation of acetylated low density lipoprotein (Asahara et al. 1999). The EPC differentiation was associated with progressive modification of surface marker expression. Several studies have identified three markers characterizing functional endothelial precursor cell: CD133, CD34, and the vascular endothelial growth factor receptor (VEGFR-2), also termed kinase insert domain receptor (KDR) or fetal liver kinase (Flk-1) (Gehling et al. 2000, Peichev et al. 2000). The CD34 antigen is a marker not exclusive of hematopoietic stem cells because it was found at extremely low concentrations on mature endothelial cells. CD133 is an early hematopoietic stem cell marker expressed on hematopoietic stem and on progenitor cells from human bone marrow but it is missing on mature endothelial cells and monocytes and it is an important marker for the identification of EPCs (Handgretinger et al. 2003). The number of CD133 cells in non mobilized peripheral blood has been reported to be in the range of 0.002% from total peripheral mononuclear cells, but the stimulation with granulocyte colony stimulating factor causes an 5-fold to 10-fold increase *in vivo* (Peichev et al. 2000). CD133-positive cells are able to differentiate *in vitro* in endothelial cells in the presence of VEGF and other growth factors and a small subset of $CD34^+$ cells from different sources express both CD133 and VEGFR-2 markers (Gehling et al. 2000). In the bone marrow, early progenitor and endothelial phenotype cells that express CD133/CD34/VEGFR2 but not vascular endothelial (VE-) cadherin and von Willebrand factor are localized (Yoder 2012). Conversely, in the peripheral blood early EPCs express CD34, CD133 and the VEGFR-2 while the late EPCs lost CD133 and began to express endothelial lineage cell markers such as von Willebrand factor, endothelial nitric oxide synthase and Ve-cadherin (Figure 6 and Table 1). Early and late EPCs are characterized by different proliferation potential. Indeed, the early-EPCs show a low proliferative ability, appear within 4-7 days of culture with a spindle-shaped and express CD14 (monocytic marker) and CD45 (pan-leukocytic) antigens. Late-EPCs develop after 2-3 weeks of culture, express the endothelial nitric oxide synthase and display a higher proliferative potential compared to early-EPCs (Resch et al. 2012, Yoder 2013, Basile et al. 2014). Mature endothelial cells of large vessels are negative for CD34 and express high amounts of VEGFR-2, VE-cadherin, and von Willebrand factor. CD133 is not detectable on the surface of mature human endothelial cells and monocytic cells (Casamassimi et al. 2007).

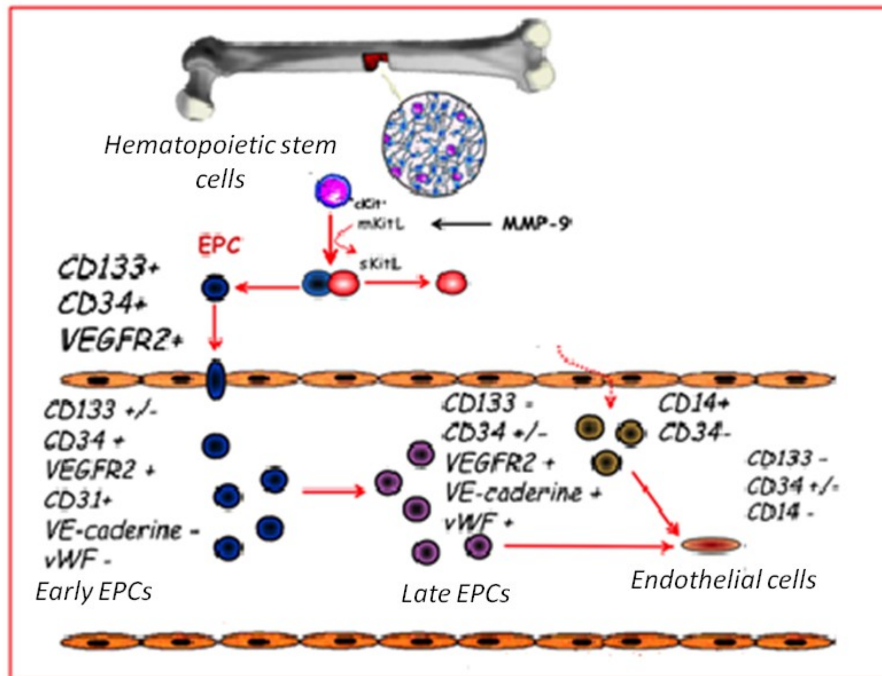

Figure 6. From bone marrow to blood circulation of EPCs. Differential expression of markers during the mobilitation and maturation of EPCs.

Table 1. Phenotipic characterization of EPCs.

CELLS	SITE OF LOCALIZATION	MARKERS
BM-EPC	Bone marrow	$CD133^+$, $CD34^+$, VEGFR-2^+
"Early" EPC	Peripheral blood	$CD133^{+/-}$, $CD34^+$,VEGFR-2^+ $CD31^+$, $CD14^+$, VE-$Caderine^+$, vWF^+
"Late" EPC	Peripheral blood	$CD34^+$,VEGFR-2^+,$CD31^+$, VE-$Caderine^+$, von Willebrand factor $^+$, endothelial nitric oxide synthase

1.3.3 EPCs and *B. henselae*

Salvatore (2008) has demonstrated for the first time that *B. henselae* can adhere and invade human EPCs both *in vivo* and *in vitro* (Figure 7) (Salvatore et al. 2008). The *in vitro* assays have revealed that the infection of EPCs with *B. henselae* impairs functional activity and significantly reduces the number of these cells. *B. henselae* adheres to EPCs through cytoplasm protrusions of cell membrane followed by internalization of bacteria detectable in vacuoles. EPCs infected show a changed cell morphology with mitochondria disruption and expansion of endoplasmatic reticulum. Interestingly, the treatment of EPCs

with L-arginine results in a reduced number of intracellular invasomes, intact cytoplasmatic mithocondria and a reduced expansion of smooth endoplasmatic reticulum. Taken together, these results support the concept that, like HPCs, EPCs can carry *B. henselae* in other organs such as endothelium in which the vasoproliferative disorders occur with the involvement of several pathways including immune response genes and de-regulation of inflammatory gene networks (Salvatore et al. 2008, Costa et al. 2010). Moreover, EPCs of Down syndrome patients have shown a higher susceptibility to infection with *B. henselae* compared to euploid cells as well as a reduced number and altered morphology, thereby confirming the angiogenesis and immune response deficit observed in Down syndrome individuals (Costa et al. 2010).

In a recent study, in order to evaluate the effect *in vivo* of *B. henselae,* a murine model of infection was used (Sommese et al. 2012). The authors have evaluated the effects *in vivo* and *in vitro* of coinfection between *B. henselae* and another gram negative bacterium, *Bacteroides fragilis*. *B. fragilis* is a component of the gut microflora of most mammals that mediates its effects through the capsular polysaccharide (PSA). *B. fragils* and the mutant ∆PSA that lack PSA component, are able to infect human EPCs *in vitro*. However, in the early EPCs coinfected with both bacteria, *B. fragilis* competes with *B. henselae* and it impairs the infection with *B. henselae*. Conversely, in the coinfection with *B. fragilis* ∆PSA the presence of *B. henselae* in EPCs is more relevant. Thus, *B. fragilis* and *B. fragilis* ∆PSA are both able to infect human EPCs but the presence of the PSA is necessary in order to compete with *B. henselae* internalization. The infection with *B. henselae* causes a drastic reduction of EPCs in mice. This reduction can be prevented in mice coinfected with *B. fragilis* but not with *B. fragilis* ∆PSA (Sommese et al. 2012).

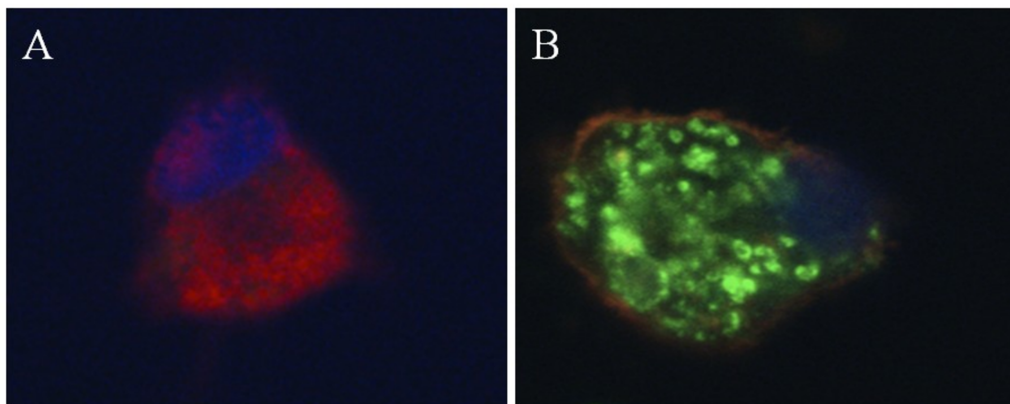

Figure 7. Representative images by immunofluorescence. Confocal image of human EPCs uninfected (A) and *B. henselae* infected (B) (by courtesy Dr. A. Casamassimi).

1.4.0 HLA and infections

The susceptibility to infections and to several human diseases such as diabetes and heart diseases depends to a complex interaction between environmental and host genetic factors. In particular, the genes involved in the immune response reflect the evolutionary advantages of a diverse immunological response to a wide range of infectious pathogens and they can contribute to the susceptibility to human diseases (Alves et al. 2006). Recently, there has been much interest in the genetic markers that through the interaction with other factors can influence the immune response to an infectious disease. Moreover, infectious agents are able to cause different immune response in different infected individuals (Bleckwell et al. 2009). In general, many genetic loci can influence the susceptibility or the resistence to infectious disease including the Human Leukocyte Antigens (HLA) system. During the immune response versus a pathogen, the HLA molecules must link the peptides derived from the pathogen and the repertoire of T cells that can be triggered by this HLA-peptide association (Klein and Sato 2000).

1.4.1 Organization of HLA complex

HLA complex is located on the short arm of chromosome 6 and contains a large number of genes that are divided in three classes. HLA class I and class II molecules are controlled by two different regions highly complex and polymorphic, including multiple loci and different alleles. HLA class I includes the molecules HLA-A, -B,-C, while HLA class II the molecules HLA-DR,-DQ and –DP, the HLA class III does not encodes HLA molecules (Klein and Sato 2000) (Figure 8).
HLA class I and II regions are components of the immune response with a high polymorphism. Indeed, there are currently 12,672 HLA alleles described by the HLA nomenclature at all loci, included in the IMGT/HLA Database and recognized in the official sequences for the WHO Nomenclature Committee for factors. In particular, about 9000 different alleles are recognized for class I and 3000 for the class II. HLA genes are closely related and are inherited in a Mendelian manner as haplotype from each parent (Table 2).

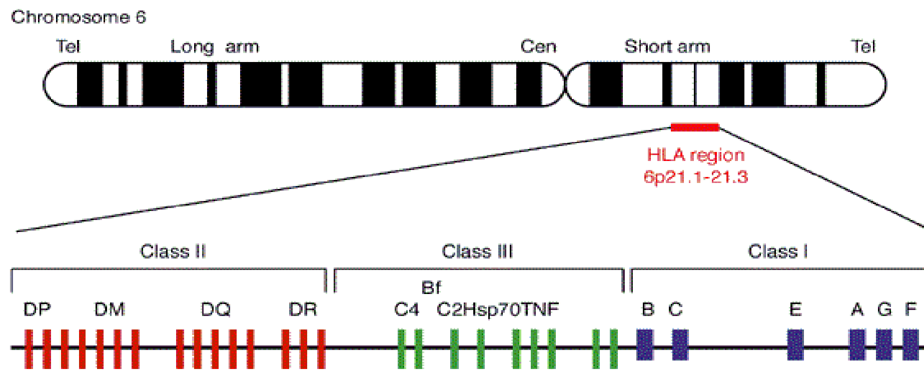

Figure 8. Classical scheme of HLA complex on chromosome 6. HLA class I region includes HLA-A,B,C genes, while class II the HLA-DR,DQ.

HLA class I molecules are expressed on the surface of all nucleated cells, class II molecules are expressed on B cells, antigen-presenting cells and T cells activated. HLA class I antigens are heterodimeric molecules divided in three regions. The extracellular region shows three domains (α1, α2 and α3) and the high polymorphic α1 and α2 domains contain variable amino-acid sequences and are the site of peptide-binding domains.

Class II molecules consist of two trans-membrane polypeptides, α and β chains and the high polymorphism of these molecules occur mostly in β chain than α. The domain α1 and α2 form an antigen-binding groove (Figure 9).

The class I and II molecules bind different peptides. In particular, class I-restricted T cells recognize endogenous antigens including cellular, transformed or pathogen proteins and the complex HLA molecule-peptide are recognized by T-cells receptor of $CD8^+$ cells. Conversely, class II molecules recognize extracellular exogenous proteins after endocytosis and degradation and are recognized by T-cell receptor of $CD4^+$ cells. The HLA antigens are characterized at serological level, by identifying the class I antigens on the T cells isolated from peripheral blood lymphocytes and the class II antigens on the B cells. However, the serologically indistinguishable variants of HLA antigens, due to few amino-acid substitutions are identified at molecular level (Klein and Sato 2000).

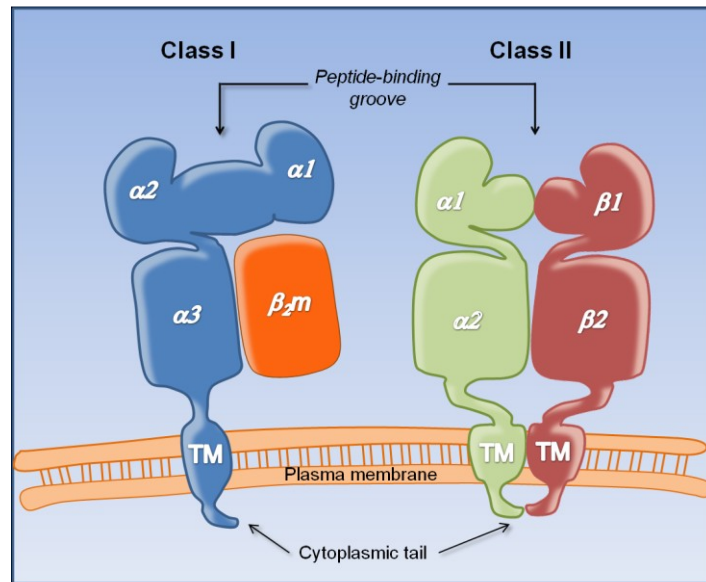

Figure 9. Structural organization of HLA class I and II molecules. Class I molecules consists of the trans-membrane polypeptide while Class II molecules consist of two trans-membrane polypeptides, α and β chains

The high polymorphism of HLA complex is localized in the antigen-binding groove, indeed some variations of amino-acids can affect the peptide-binding specificity of HLA molecules. In the different populations and geographic regions there are a different distribution and frequency of HLA molecules due to the function of these molecules of to present infectious agents. To date, the number of known alleles increase thank to the modern molecular techniques of sequence-based typing. Several new alleles are detected in the exons encoding the domain that present the antigens but also in others intron or exon domains (Table 2).

Table 2. The most important HLA-A, B and C loci involved in the self/no-self recognition and in the immune response and the related antigen and allele numbers.

Loci		N° antigens	N° alleles
HLA class I	**HLA-A**	**24**	**2995**
	HLA-B	**49**	**3760**
HLA class II	**HLA-DR**	**20**	**1700**

1.4.2 Effect of HLA polymorphism on infectious disease

The polymorphic HLA alleles can promote the genetic diversity and the variable susceptibility of the different individuals to the diseases. The HLA molecules are involved in the presentation of antigens and for this reason,

individuals presenting a certain combination of HLA alleles that not binds in a proper manner to antigens or that not triggers an adequate response will be less suitable to resist to the pathogens. The HLA alleles that provide protection are able to select and stimulate immune cells in order to destroy the infected cells. A positive or negative genetic correlation between classical HLA loci and major infectious disease was found. Some of these associations have been investigated by showing a strong correlation, while others not much. The interaction between factors involved in immune response to infection including the intensity of the infection or the function of T cells and genetic factors such as HLA complex can determine the evolution of the disease (Alves 2006).
Different mechanisms of HLA/disease association were proposed such as the greater or less affinity between a particular HLA molecule and the peptide. In addition, the HLA antigens can act as receptors for pathogens by promoting or avoiding the cell entry. HLA can influence the process of thymic selection by promoting the tolerance of T cells versus the pathogens due to a molecular mimetism between pathogens and host antigens (Kulkarni et al. 2008).
Numerous evidences are focused on the association between HLA and viral infections such as HCV, HBV and HIV demonstrating a protective or the causative role of some HLA molecules versus the onset of viral infections (Elahi and Horton 2012, Grimaldi et al. 2013). However, the association between HLA and mycobacterial, bacterial and parasitic infections has been reported and emphasized (Blackwell et al. 2009, Alves et al. 2006). Several studies have demonstrated an association between mycobacterial infections and HLA. The consensus in the literature is that HLA-DRB1*15, HLA-DRB1*16 and HLA-DR3 are associated with the tuberculoid leprosy form while HLA-DQB1*01 is associated with the lepromatous leprosy form. Moreover, it was found that HLA-DRB1*15 allele is associated with a severe form of tubercolosis and with the drug-resistant, while a protective association was observed between some form of leprosy and HLA-DQB1*02 (de Souza-Santana et al. 2015). Mohammed (2014) has reported a high HLA frequency of B*18 and DRB1*01 and a decrease of B*51 in patients with mycobacterium tuberculosis infection (Mohammed et al. 2014). The association of bacterial infection with ankylosing spondylitis, anterior uveitis is common. It was found that Klebsiella antigens are similar to HLA-B27 antigens and through a mimetism mechanism they can promote the ankylosing spondylitis onset as well as the age and the frequency at which it occurs (Shamji et al. 2008). In patients with anterior uveitis, the association between Gram-negative bacteria including Serratia, Salmonella and *B. henselae* and HLA-B*27 was described. In addition, an association with HLA-DR antigens in patients negative for HLA-B27 it was found. Furthermore, Chlamydia, Ureoplasma, Shigella infection are related to HLA-B27, HLA-B51 and HLA-B35. High IgG levels antibody against *B. henselae* was identified in patients with uveitis B-27 positive. Kerkhoff (2000) has reported the frequency of HLA-B27 in patients with uveitis and serological characteristics of acute infection with *B. henselae* (Kerkhoff and Rothova 2000).

2. Aim of the study

Infectious complications represent a significant source of morbidity and mortality in HTx recipients. However, the importance of infection in post-transplantation time it is well know whereas there are few studies evaluating the incidence and the types of infection in patients awaiting HTx and the impact on the early post-transplantation period when the immunosuppressive therapy predisposes the patients to infectious complications. These infections are due to pathogens that rarely cause infection in immunocompetent individuals and can be difficult to identify, to treat, and can be lethal in the immunosuppressed patients. Most post-transplantation infections represent reactivation of latent or quiescent infections that had been contracted prior the transplantation. Recently, several papers have showed the incidence of *B. henselae* infections in solid organ transplantation recipients, where these bacteria cause a disseminated infection with involvement of different organs mainly due to vasoproliferative lesions. Furthermore, i*n vitro* and *in vivo* studies data have demonstrated that isolated EPCs are able to internalize *B. henselae.* Susceptibility to infections depends on the complex interaction of environmental and host genetic factors such as the HLA molecules. Many studies have demonstrated a statistical association between HLA class I and II molecules and susceptibility or protection to a range of complex infectious diseases and most of these studies have used a case-control approach.

This study aims to evaluate in patients awaiting HTx enrolled in the Regional Reference Laboratory of Transplant Immunology of Second University of Naples, the seroprevalence of *B. henselae,* the quantitative analysis of EPC cells and the correlation between *B. henselae* prevalence and HLA antigens.

Moreover, in order to investigate this unique human pathology, we evaluate the effects of B. henselae infection in an immunocompromised murine model by analyzing the tissue damage and the number of EPC circulating. Immunocompromised mice can represent a useful model in order to evaluate the B. henselae infection because patients awaiting transplantation will be underwent immunosuppressive therapy in the post-transplantation time.

3. Materials and Methods

3.1 Patients and controls

Patients were enrolled at the Laboratory of Transplant Immunology of Second University of Naples and, serum samples of 38 patients awaiting HTx and of 50 volunteers (control group) were collected. EDTA-blood was obtained from all control subjects and patients. Following blood collection, sera were separated by centrifugation and stored at -20°C. The patients selected and enrolled in the waiting list for HTx have severe symptoms of heart failure, angina or rhythm disturbances intractable and they are in severe clinical conditions (ejection fraction < 30% and peak oxygen consumption max < 8 ml/kg/min) with no alternative form of treatment available, other than a HTx. Once a patient with severe congestive heart failure has been identified as having a limited life expectancy and severely impaired quality of life, cardiac transplantation should be considered. Irreversible pulmonary hypertension, active infection and cancer are criteria that can prevent a person from being eligible for transplantationation

Exclusion criteria for control group were any clinical history of lymphadenopathy and/or fever in the last 6 months. Patients and controls were normalized for cat exposure. None of individuals included in this study presented clinical symptoms. Written informed consent was obtained from patients and volunteers according to the Declaration of Helsinki.

3.2 Serological testing for *B. henselae* antibody detection

A commercially available *Bartonella* Indirect Immunofluorescent Assay (IFA) (Focus Diagnostics, Cypress, California, U.S.A.) was used to assess the qualitative detection and semi-quantitation of human serum IgM and IgG antibodies to *B. henselae* and *B. quintana* according to manufacturer's instructions. In the first stage, suitable dilutions of patient sera were added to appropriate slide wells in contact with the substrate for IgM and IgG, respectively. In the second stage, fluorescein-labeled antibody to IgM or IgG, was added, respectively. After the slide was washed, dried, and mounted, it was examined by fluorescence microscopy (Zeiss West, Germany) at magnifications of 40X and 100X. Each kit included positive and negative controls. Positive reactions appeared as bright apple-green fluorescent bacteria. According to the recommendations of the manufacturer titers from 1:20 for IgM and 1:64 for IgG were considered positive.

3.3 Serological and molecular HLA typing

All patients were tested for HLA class I (HLA-A and -B) and class II (-DR) loci by serological and molecular HLA typing. The serological test was performed by standard complement-dependent microlymphocytotoxicity assay

Mononuclear cells of both patients and control group were isolated from peripheral blood using magnetic monodisperse microspheres (Dynabeads TM-Invitrogen, Italy) coated with monoclonal antibodies specific for isolation of B and T lymphocytes. The cells, attached to the microspheres, were subsequently used in HLA typing plates with acridin orange/ethidium bromide to stain viable and dead cells. Genomic DNA was extracted from whole peripheral EDTA anticoagulated blood by commercial DNA isolation kit (Promega, Italy). Molecular typing was carried out by Sequence-Specific Primed PCR (PCR-SSP) method using a micro SSP-HLA A-B-DR generic DNA typing tray (Olerup SSP ABDR, Biospa, Italy). All patients are typed only for HLA- A-B-DR loci at 2 digit. According to the manufacturer's instructions, the PCR was performed by using the Gene Amp PCR system 9700 (Applied Biosystems, Foster City CA, USA). The PCR products were separated on 2% agarose gel containing ethidium bromide and visualized under ultraviolet (UV) light. (Grimaldi V 2013).

3.4 Flow cytometry detection of human circulating EPCs

The total number of circulating EPCs was analyzed by flow cytometry (Casamassimi 2007). 100µl of EDTA-blood samples of controls and patients were incubated 30 minutes in the dark with the following antibody combinations: phycoerythrin (PE)-conjugate-anti-CD133\2 and Allocyanin-conjugated (APC)- anti-KDR-2 (Miltenyl Biotec, Gerrmany). A fluorescent isotype identical antibody control was added to the blood sample and incubated in the dark at room temperature for 15 minutes. Red blood cells were lysed by the addition of 2 ml of Pharmalyse TM solution (BD sciences) followed by incubation in the dark at room temperature for 15 min. FACS analysis was performed using BD FACSAnto II and data analyzed using DIVA software. Each analysis includes n°10000 events in the lymphocyte region. Three samples replicates from each patient were analyzed and the average was performed.

3.5 Bacterial strains, growth conditions and mice infection

The *B. henselae* strain ATCC49882 (LGC Promochem) was stored at -80°C in tryptone soya broth (TSB) until use and grown on Columbia agar with 5% defrinated sheep blood at 37°C and with 5% of CO_2.
SCID/Beige 8 weeks of age, were purchased from Charles River Laboratory.. Mice were infected with *B. henselae* via intraperitoneal route at dose of 10^9 CFU/mouse suspended in TSB. Control mice were injected with the same medium in the absence of bacteria. Following infection, mice were observed daily for signs of illness. After 36 days of infection, blood was withdrawn by cardiac puncture before sacrifice and collected in heparinized tubes. At the times of euthanasia, liver, spleen and aorta were excised and fixed for microscopic analysis.

3.6 Histopathology

All samples (liver, spleen and aorta) for immunohistochemistry were fixed in formaldehyde (4%), embedded in paraffin and sliced into 5 mm thick sections were cut; one slide was stained with haematoxylin-eosin and the remaining were used for IHC analyses, Masson Trichome and Warthin-starry stain using standard protocols (Sommese 2012, Chiaroviglio 2010). After de-paraffinization, slides containing two serial sections were processed for heat-induced epitope retrieval. When cooled, slides were washed in PBS and then incubated for 609 at 37uC with the primary antibody. Arterial segments, livers and spleens were stained with a mouse monoclonal antibody anti-B. henselae diluted 1/100 (Novus Biologicals). After a further wash in PBS, sections were incubated with the secondary antibody biotinylated for 15 minutes at 37°C.

3.7 Transmission Electron Microscopy

The aorta and liver tissues were cut into 1 mm3 blocks and fixed in 1.5% glutaraldehyde in 0,067 M cacodylate buffer at pH 7.4 (Wisse et al. 2010). The samples were left in fixative for 3 h at 4°C and then washed in 0,134 M cacodylate buffer, pH 7.4, at 4°C. Then they were post-fixed in 1% osmium tetroxide in 0,067 M cacodylate buffer, pH 7.4, at 4°C for 1 h and after washed in 0,134 M cacodylate buffer, pH 7.4, at 4°C. Fixed tissues were dehydrated in ascending series of ethyl alcohol and then embedded in epon. Semi-thin (1,5 mm) sections of transmission electron microscopy embedded liver and aorta samples were cut with a glass knife for light microscopic observations. Sections were stained with 1% toluidine blue solution prepared in 1% sodium tetraborate buffer. Ultra-thin (50–80 nm) section were cut and stained first with uranyl acetate 3% in 50% ethyl alcohol and then with 2,6% lead citrate. These sections, loaded on 200 mesh grids, were observed in a JEM-1011 (JEOL) transmission electron microscope at 100 kV.

3.8 EPC number in infected mice

A single blood aliquot was collected by cardiac puncture from the ventricle under anaesthesia, with Tribromoethanol 240 mg/Kg, using appropriate size needles and collected into heparin-coated tubes. A volume of 100 μl of blood was incubated for 30 minutes at 4°C with Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody PE-anti-Sca-1 (phycoerythrin-conjugated stem cell antigen-1) and APC-anti-Flk-1 (allophycocyaninconjugated fetal liver kinase-1) (BD Pharmingen). After incubation, erythrocytes were lysed using FACS lysing solution (Becton-Dickinson) 2 ml for each tube for 10 minutes at room temperature at dark. After the lysis, two washes were performed with PBS by centrifugation at 1900 rpm for 10 minutes. Isotype-identical IgG antibodies were used as controls (BD Pharmingen). The samples were analyzed on a FACScan (Becton-Dickinson)

using DIVA software. Each analysis included approximately 10000 events, referred to the total number of white cells. The samples were analyzed in duplicate

3.9 Data analysis

The baseline clinical and laboratory characteristics of the patients in the two groups were compared with continuous variables evaluated using a one-way ANOVA and categorical variables were analyzed using the Chi square.
Statistical analysis of HLA typing was carried out by SPSS (statistical package of social science) version 17 (SPSS Inc., Chicago, IL, USA). Data were expressed in terms of frequencies and percentage. Chi square was used as a significance test for comparison between the study groups. Multiple logistic regression analysis was used to evaluate HLA alleles associated with B. henselae infection. In the model statistically significant variables were included in univariate analysis. Odds Ratio (OR) and 95% Confidence Interval (CI) were calculated. Significance was considered when p value was less than 0.05.

4. Results

4.1.1 Clinical data

In order to evaluate the seroprevalence of *B. henseale* in patients awaiting HTx respect to a control population represented by volunteers, enrolled at the Laboratory of Transplant Immunology of Second University of Naples, we have collected sera of 38 patients and 50 controls. All the subjects analyzed living in Campania Region.

The patients selected and enrolled in the waiting list for HTx have severe symptoms of heart failure, angina or rhythm disturbances intractable and they are in severe clinical conditions (ejection fraction $< 30\%$ and peak oxygen consumption max<8 ml/kg/min) with no alternative form of treatment available, other than a HTx. Indeed, once a patient with severe congestive heart failure has been identified as having a limited life expectancy and severely impaired quality of life, HTx should be considered. Irreversible pulmonary hypertension, active infection and cancer are criteria that can prevent a person from being eligible for transplantation.

In Table 3 we reported the baseline demoghraphic and clinical characteristics of study subjects.

Table 3: Baseline data of patients awaiting HTx

Gender, n (%)	
Male	28 (73.7)
Female	10 (26.3)
Age, y	52±16
Cardiac disease, n (%)	
No-ischemic DCM	21 (55.3)
Ischemic DCM	17 (44.7)
Valve implant, n (%)	
Yes	6 (15.8)
No	32 (84.2)
Diabetes, n (%)	
Yes	7 (18.4)
No	31 (81.6)
Hypercolesterolemia, n (%)	
Yes	19 (50)
No	19 (50)
Hypertension, n (%)	
Yes	10 (26.3)
No	28 (73.7)
Statin use, n (%)	
Yes	19 (50)
No	19 (50)
Renal failure, n (%)	
Yes	18 (47)
No	20 (53)

In particular, the patients enrolled in waiting list for HTx were affected by ischemic or no-ischemic dilated cardiomyopathy (DCM), 55.3% and 44.7% patients, respectively. The mean age of the 38 study patients (28 male and 10 female) was 52±16 years. The 15,8% of patients present cardiac valve implantations, the 18,4% are affected by diabetes, the 50% of patients had incidence of hypercholesterolemia and the 10% of patients had hypertension. The mean age of control population (30 male and 20 female) was 38±8 years.

4.1.2 Serological analysis of *B. henselae* antibody

Serum samples of 38 patients awaiting HTx and 50 healthy donors were examined by IFA. In our study, we have found a *B. henselae* antibody significant positivity rate of 21% (n=8) in patients awaiting HTx (p=0.002) (Table 4). Moreover, in our patients the seroprevalence of *B. henselae* was not significantly associated with age, gender, ischemic and non-ischemic DCM and other clinical parameters (p>0.05). The differences in comorbidity between cases and controls were statistically different (1.41±0.96 vs 0.42±0.32; p=0.001). Moreover, the age of the patients in waiting list for HTx was higher than control group (52.1±17.0 vs 38.8; p=0.001). The prevalence of comorbidities evaluated (dilated cardiomyopathy, valve disease, renal failure, diabetes, and hypertension) and the differences between cases and controls are reported in Table 4.

Table 4 Seroprevalence of *B. henselae* in patients awaiting with advanced heart failure awaiting HTx and in control subjects

Variables	Patients # 38	Controls # 50	*p*
Age (years)	52.1±17.0	38.0±8.0	0.001
Comorbidity	1.41±0.96	0.42±0.32	0.001
B. henselae antibodies (%)	21	0	0.002
Valve Diseasee (%)	16.2	2.0	0.016
Previous myocardial infarction (%)	37.8	0	0.001
Diabetes (%)	18.9	0	0.001
Hypertension (%)	24.3	8	0.005
Renal Failure (%)	47.1	0	0.000

In detail, we found a positive rate of 8% (p>0.05) for IgM antibody against *B. henselae* and a significant value of 13% (p=0.02) for IgG antibody. All the

controls analyzed are resulted negative both for IgM and IgG antibodies against *B. henselae*.

In Table 5, we report in detail the *B. henselae* seropositivity rate of patients awaiting HTx (n=8) that are resulted positive to IgM or IgG antibodies. The highest antibody dilution with a positive reaction was indicated. We found a positive rate of 8% (n=3 patients) with $p>0.05$ for IgM antibodies, while a significant value was found for IgG with 13% (n=5 patients) of positivity ($p=0.02$). According to the recommendations of the manufacturer and to the literature data, titers from 1:20 for IgM and 1:64 for IgG were considered positive. In Table 5, also demograpich and clinical characteristics of patients are reported. All controls resulted negative for both IgM and IgG antibodies and they have shown only a low percentage of hypertension and mitral prolapsed.

Table 5 Seropositivity of *B. henselae* IgM and IgG antibodies in patients awaiting HTx

Gender	Age	IgM anti-*B. henselae*	IgG anti-*B. henselae*	Heart disease DCM	Valve	Diabete	Hyper-cholesterolemia	Hypertension	Transfusion
F	8	//	1:128	no-ischemic	Yes	No	No	No	Yes
M	64	1:20	//	ischemic	No	Yes	Yes	Yes	No
M	50	//	1:128	ischemic	No	No	Yes	Yes	No
M	67	//	1:64	ischemic	No	No	Yes	No	Yes
M	55	//	1:64	no-ischemic	Yes	No	No	No	No
M	33	1:20	//	ischemic	No	No	Yes	No	Yes
M	66	//	1:64	ischemic	No	No	Yes	Yes	No
F	56	1:20	//	no-ischemic	No	No	No	No	No

The Fig. 10 reports the representative IFA images of one patient resulted positive to *B. henselae* IgG (A) and a negative control of *B. henselae* IgG (B). None of individuals included in this study showed positive reactions to *B. quintana*. All patients positive for IgG and IgM antibodies, were monitored at least twice at different sampling times confirming the result. In particular, we have also found in the live patient positive for IgM, reported in the last line of Table 5, after two months of positivity for IgM the seroconversion of antibodies in IgG with titre of 1:64.

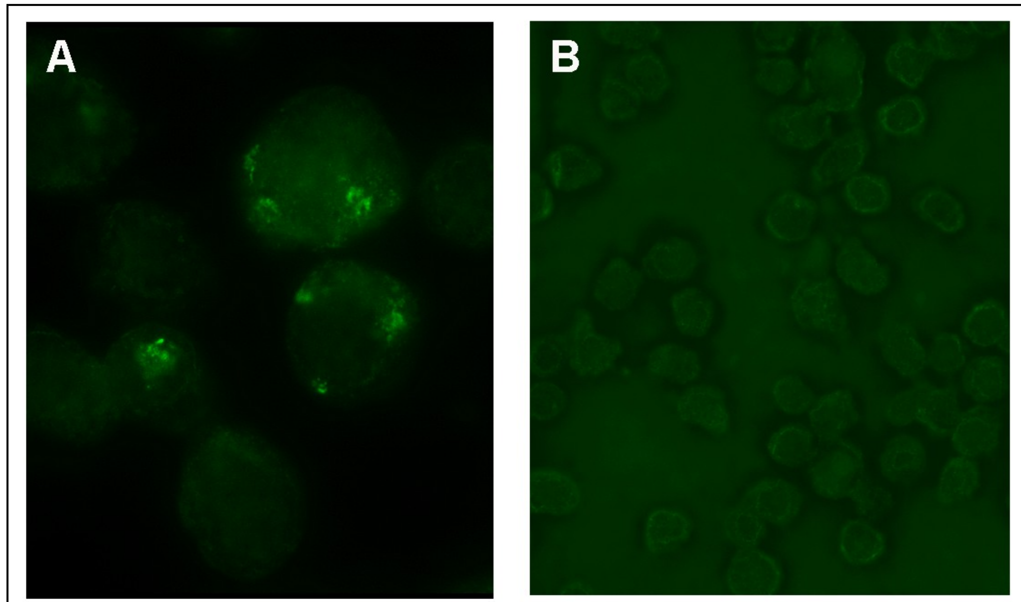

Figure 10 Immunofluorescent assay images. (A) Serum sample of one patient awaiting HTx positive to *B. henselae* IgG, magnification 100X; (B) negative control of *B. henselae* IgG supplied by the manufacturer, magnification 40X. (in collaboration with Dr. C. Pagliuca).

4.2 Circulating EPC detection

The detection of EPCs in peripheral blood samples has been performed by flow cytometry analysis that represents the gold standard for this aim because it is a method simple and reproducible. At this regard, based on the description of EPCs, a combination of markers including at least a marker of stemness usually CD133 and a marker of endothelial commitment such as KDR (also known as VEGFR-2 and Flk-1) were used. In particular, an phycoerythrin (PE)-conjugate-anti-CD133 and an allophycocyanin (APC)-conjugated KDR were used. Each analysis included 10.000 events. EPCs are defined as double-positive $CD133^{+}/KDR^{+}$.
The numbers of circulating EPCs were gates with lymphocytes as indicated by the arrow in the inserted forward and sideward scatter plot image in figure 11A. We used an isotype control in which no positive events are obtained in Q2-1 area (Figure 11B). In the figure 11C we reported a representative sample with double-positive marked cells in the Q2-1 area corresponding to EPCs.

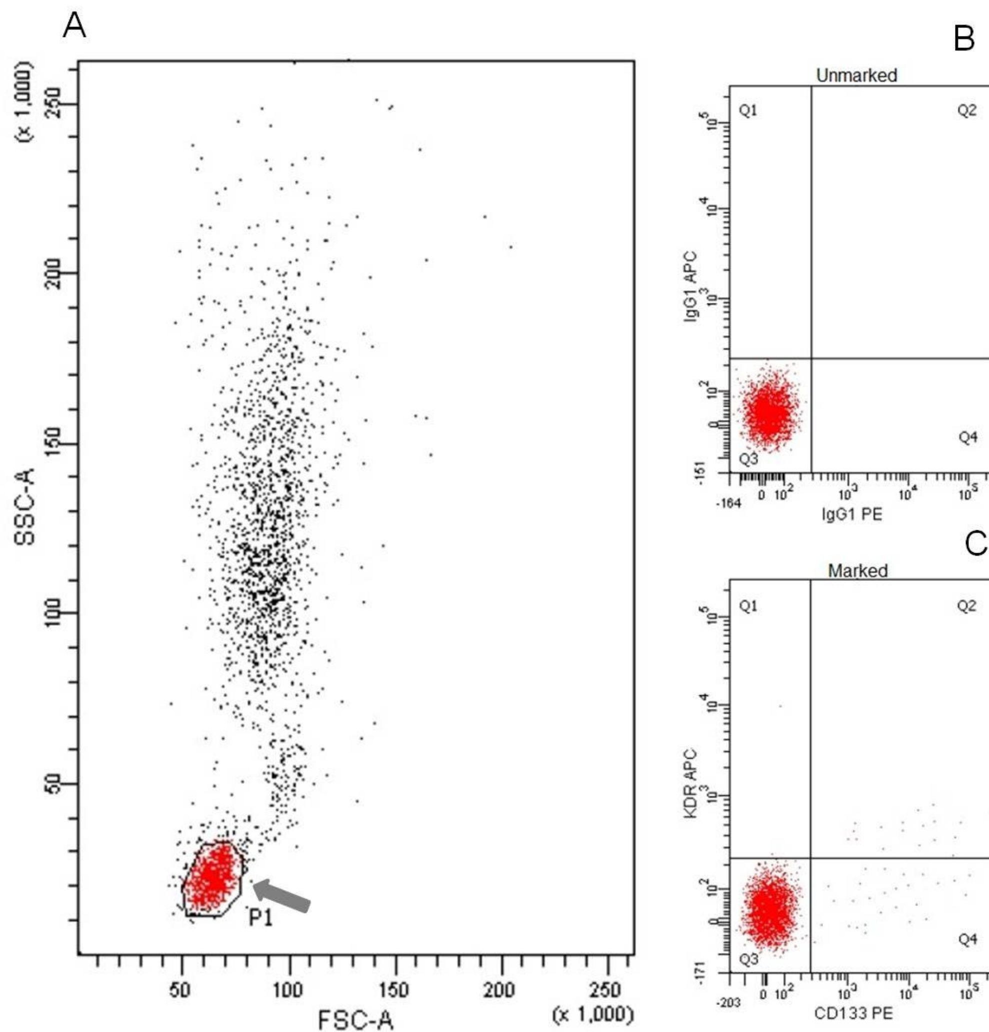

Figure 11. Example of forward and sideward scatter plot image cytometric analysis for the assessment of EPCs. Delineation of the population lymphocytes (A) in the sample, as indicated by an arrow.
Representative identification of subpopulation in the sample marked with the isotype control (B) and with $CD133^+/KDR^+$ (C).

EPC circulating number has shown a decrease of 20% in patients awaiting HTx *B. henselae*-negative respect to control group, whose EPC number was considered 100% (1.0) but this difference is not significant ($p>0.05$). The number of EPCs in patients *B. henselae*-positive was further reduced respect to control (42%). Interestingly, EPCs number were significantly lower in patients with *B. henselae*-positive patients respect to negative ($p <0.05$) with a reduction of 22% (Figure 12).

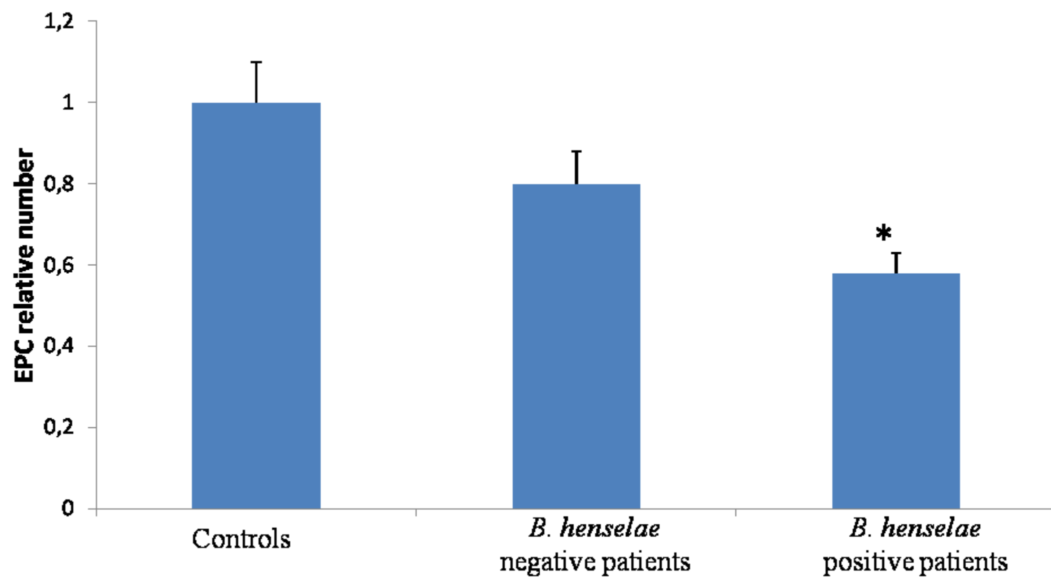

Figure 12. EPC number in control group, *B. henselae* negative and *B. henselae* positive patients. EPC number was measured by FACS analysis. KDR+/CD133+ double positive cells were considered as human EPCs (*p<0.05).

4.3 Distribution of HLA-A,-B,-DR alleles in patients awaiting HTxat and association with *B. henselae* infection

The possible association between HLA class I and class II alleles and *B. henselae* infection were assessed by comparing the frequencies of these alleles in two groups of patients, negative and positive for *B. henselae*.
We have performed a serological and molecular typing at low resolution of HLA-A, B and –DR loci of all patients awaiting HTx enrolled in our study. The serological typing allows to identify the HLA antigens expressed on the membrane of T and/or B cells, while the molecular typing identified all the alleles HLA including the antigens that are null and for this reason not expressed or expressed at low levels on the cell membrane.
The distribution of HLA-A* alleles among all *Bartonella*-positive patients versus *Bartonella*-negative patients is reported in Table 6A.

Table 6 A. Comparison of HLA-A* frequencies between *B. henselae* negative patients versus positive patients

HLA	*B. henselae* negative patients (30)		*B. henselae* positive patients (8)		OR	95% CI		*p*
	N	%	N	%				
A*01	7	23,3	1	12,5	0,47	0,05	4,50	0,51
A*02	9	30,0	4	50,0	2,33	0,48	11,45	0,30
A*03	7	23,3	3	37,5	1,97	0,37	10,40	0,42
A*11	4	13,3	2	25,0	2,17	0,32	14,71	0,43
A*23	1	3,3	1	12,5	4,14	0,23	74,70	0,34
A*24	10	33,3	1	12,5	0,29	0,03	2,65	0,27
A*26	3	10,0	1	12,5	1,29	0,12	14,33	0,84
A*29	4	13,3	1	12,5	0,93	0,09	9,69	0,95
A*30	6	20,0	0	-	-	-	-	-
A*32	3	10,0	1	12,5	1,29	0,12	14,33	0,84
A*33	2	6,7	0	-	-	-	-	-
A*69	1	3,3	0	-	-	-	-	-

OR=odds ratio; CI=confidence interval. *$p<0.05$ is significant

Among *B. henselae*-negative patients, the most frequent HLA-A* allele is A*24, while in the group of positive patients the most frequent is A*02, but no statistical significance was found between HLA-A* and *B. henselae* infection.
The distribution of HLA-B* alleles among all *B. henselae*-positive patients versus *B. henselae*-negative patients is reported in Table 6 B.

Table 6 B. Comparison of HLA-B* frequencies between *B. henselae* negative patients versus positive patients

HLA	***B. henselae* negative patients (30)**		***B. henselae* positive patients (8)**		**OR**	**95% CI**	***p***
	N	***%***	***N***	***%***			
B*07	3	10,0	0	-	ND	-	ND
B*08	4	13,3	0	-	ND	-	ND
B*13	2	6,7	0	-	ND	-	ND
B*14	1	3,3	0	-	ND	-	ND
B*15	4	13,3	1	12,5	0,93	0,09-9,69	0,95
B*18	8	26,6	1	12,5	0,39	0,04-3,71	0,41
B*35	***6***	***20,0***	***5***	***62,5***	***6,67***	***1,23-36,05***	***0,03***
B*37	1	3,3	0	-	ND	-	ND
B*38	1	3,3	0	-	ND	-	ND
B*39	3	10,0	0	-	ND	-	ND
B*40	1	3,3	1	12,5	4,14	0,23-74,70	0,34
B*41	2	6,7	0	-	ND	-	ND
B*44	3	10,0	2	25,0	3,00	0,41-22,08	0,28
B*45	1	3,3	0	-	ND	-	ND
B*49	2	6,7	1	12,5	2,00	0,16-25,34	0,59
B*50	2	6,7	0	-	ND	-	ND
B*51	5	16,7	2	25,0	1,67	0,26-10,77	0,59
B*52	2	6,7	0	-	ND	-	ND
B*55	2	6,7	0	-	ND	-	ND
B*58	3	10,0	1	12,5	1,29	0,12-14,33	0,84

OR=odds ratio; CI=confidence interval. *$p<0.05$ is significant

Among *B. henselae*-negative patients, the most frequent HLA-B* allele is B*18, while in the group of positive patients the most frequent allele is B*35. Interestingly, in univariate analysis HLA-B*35 allele results more frequent in *B. henselae*-positive patients compared to negative group (OR 6.67; 95% CI=1.23-36.05; p=0.03).
The distribution of HLA-DRB1* alleles among all *B. henselae*-positive patients versus *B. henselae*-negative patients is reported in Table 6 C.

Table 6 C. Comparison of HLA-DRB1* frequencies between *B. henselae*-negative patients versus positive patients

HLA	*B. henselae* negative patients (30)		*B. henselae* positive patients (8)		OR	95% CI	*p*
	N	*%*	*N*	*%*			
DRB1*01	0	-	1	12,5	ND	-	ND
DRB1*03	6	20,0	0	-	ND	-	ND
DRB1*07	9	30,0	2	25,0	0,78	0,13-4,62	0,78
DRB1*08	5	16,7	0	-	ND	-	ND
DRB1*09	0	-	1	12,5	ND	-	ND
DRB1*11	14	46,7	8	100,0	ND	-	ND
DRB1*12	1	3,3	1	12,5	4,14	0,23-74,70	0,34
DRB1*13	4	13,3	1	12,5	0,93	0,09-9,69	0,95
DRB1*14	2	6,7	0	-	ND	-	ND
DRB1*15	1	3,3	1	12,5	4,14	0,23-74,70	0,34
DRB1*16	7	23,3	0	-	ND	-	ND

OR=odds ratio; CI=confidence interval. *$p<0.05$ is significant

Among all patients the allele HLA-DRB1*11 is the alleles most frequent in both group of patients and no significant association was found with infection. A multiple logistic regression model, including age, gender, cardiac diagnosis of ischemic and no-ischemic DCM and significant HLA variables, confirms that a significant risk of *B. henseale* infection is present in patients with HLA-B*35 (OR=7.62; 95% CI=1.27-45,60; p=0.026). (Table 6 D.)

Table 6 D. Multiple logistic regression analysis of variables associated with *B. henselae* infection

Variables	**OR**	**95% CI**	***P value***
Age	0,99	0,94-1,05	0,740
Gender	1,20	0,12-11,58	0,876
HLA-B*35	***7,62***	***1,27-45,60***	***0,026***
Diagnosis	0,58	0,08-4,48	0,603

OR=odds ratio; CI=confidence interval. *$p<0.05$ is significant

4.4 Pathogenic effects of *B. henselae* infection in immunodeficient mice

4.4.1 Mice infection

We have developed a immunodeficient mouse model of *B. henselae* infection to investigate the effects of infection and to provide a model for human disease in immundeficient host including transplanted patients.
Three SCID/Beige mice were infected with 10^9 CFU/mouse suspended in TSB via intraperitoneal. No animals died after infection and all animals were observed and monitored during the 36 days of infection.

4.4.2 Morphological analysis of mice tissues.

Morphological study was performed by hematoxylin-eosin staining to evaluate the morphology and the inflammatory infiltrate and Masson Trichomic staining to differentiate the collagen. In the liver of uninfected mice, the morphological study have evidenced absence of inflammatory infiltrate and supporting collagen in the area of the portal triad. In animal infected with *B. henselae* we have observed the presence of moderate/severe inflammatory infiltrate as revealed by hematoxylin-eosin staining and increased of fibrosis with collagen-deposition in the area of the portal triad as showed by Masson Tricomic staining (Figure 13). The assessment of periportal and intraparenchymal inflammatory infiltrate was performed semiquantitatively (absent, mild, moderate and severe), the same parameters were used to evaluate the fibrosis. The same analysis were performed on the spleen of the two groups of animals. Morphological analysis showed an increase of fibrosis and deposition of collagen fibers in the subcapsular region, intracapsular and perivascular in mice infected with *B. henselae* (Figure 14).
In the aorta, we observed no significant differences in the intima, media and adventitia layers in the uninfected and *B. henselae* infected mice. In mice *B. henselae* infected we have found a small increase of collagen deposition in the adventitia and intima layers (Figure 15).

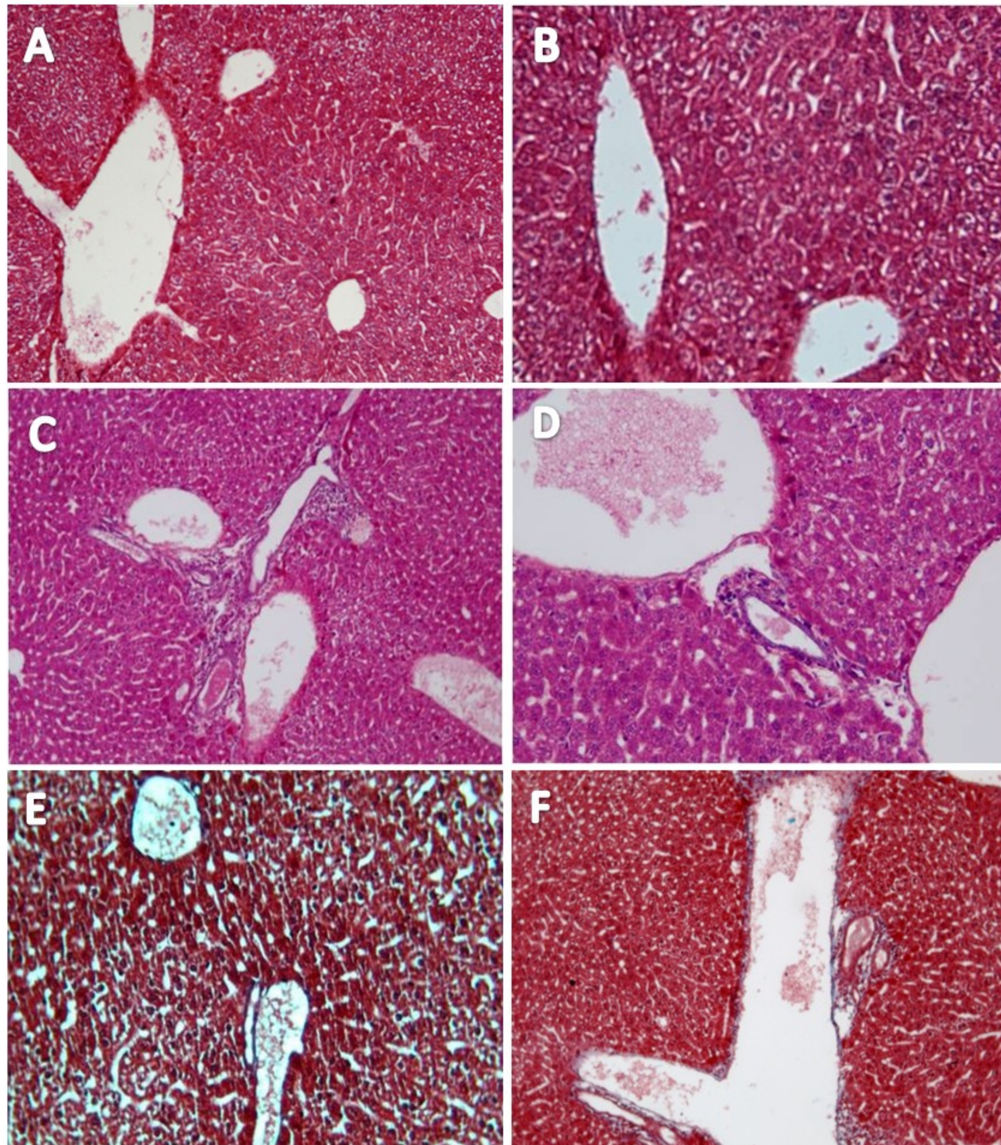

Figure 13. Representative microscope image of hematoxylin-eosin (A-D) and Trichrome staining (E, F) of liver tissue. Absence of inflammatory infiltrate in the area of the portal triad of uninfected mice at x10 (A) and x20 magnification (B) with absence or a minimal amount of supporting fibrocollagen (E, at x 20 magnification). Presence of moderate/severe inflammatory infiltrate in the area of the portal triad of *B. henselae* infected mice at x10 (C) and x20 (D) magnification. Increase of fibrosis in the area of the portal triad in *B. henselae* infected mice (F x10), (in collaboration with Dott.ssa G. Mansueto).

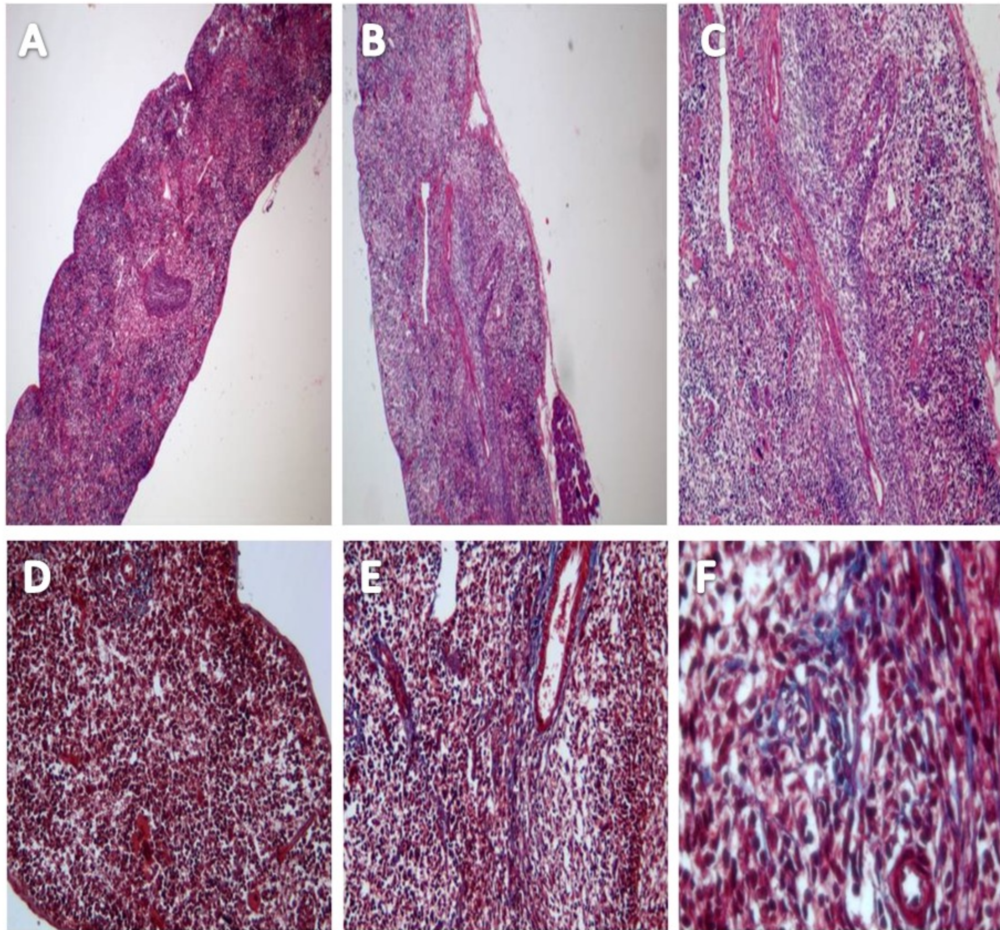

Figure 14. Representative image of hematoxylin-eosin (A-C) and Trichrome staining (D-F) of spleen tissue. Absence in the spleen of uninfected mice of collagen deposition (A, hematoxylin-eosin x4 magnification and D, Masson Thricomic x20 magnification). Significant increase of subcapsular, intracapsular and perivascular deposition of collagen in *B. henselae* infected mice (B, C hematoxylin/eosin x4 and x10 magnification and E, F Masson Thricomic x20 and x63 magnification), (in collaboration with Dott.ssa G. Mansueto).

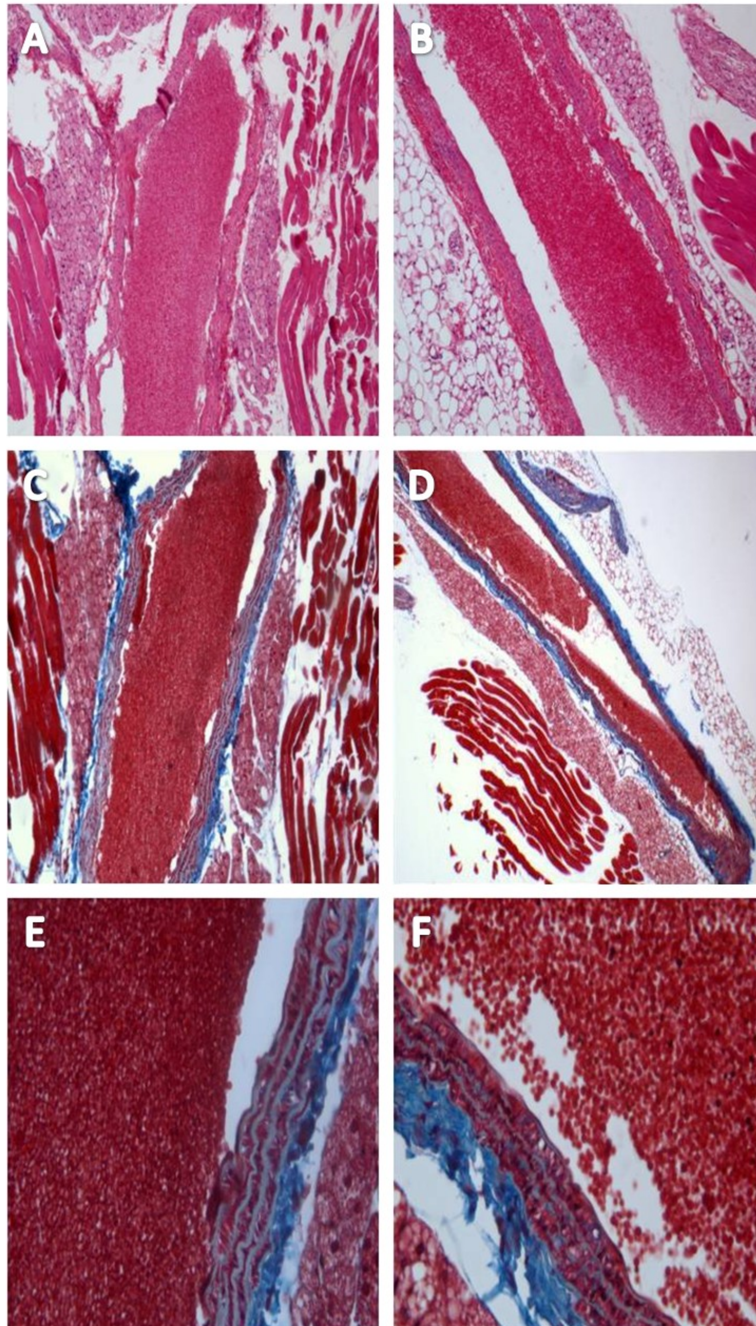

Figure 15. Representative microscope image of hematoxylin-eosin (A-B) and Trichrome staining (C-F) of aorta. Normal structure of intima and adventitia layers of aorta of uninfected mice (A, hematoxylin/eosin x10 magnification, C, E Masson Thricomic x4 and x40 magnification). Small increase of collagen deposition in the intima and adventitia of *B. henselae* infected mice (B hematoxylin/eosin x10 magnification, D, F Masson thricomic x10 and x40 magnification) (in collaboration with Dott.ssa G. Mansueto).

The lesions were further characterized using anti-*Bartonella* specific antibody and the Warthin-Starry stain, preferentially used for CSD diagnosis, in order to identify the presence of bacteria in the damaged tissues. We have identify the presence of bacteria in the liver and spleen of infected mice as reported in Figure 16 and 17.

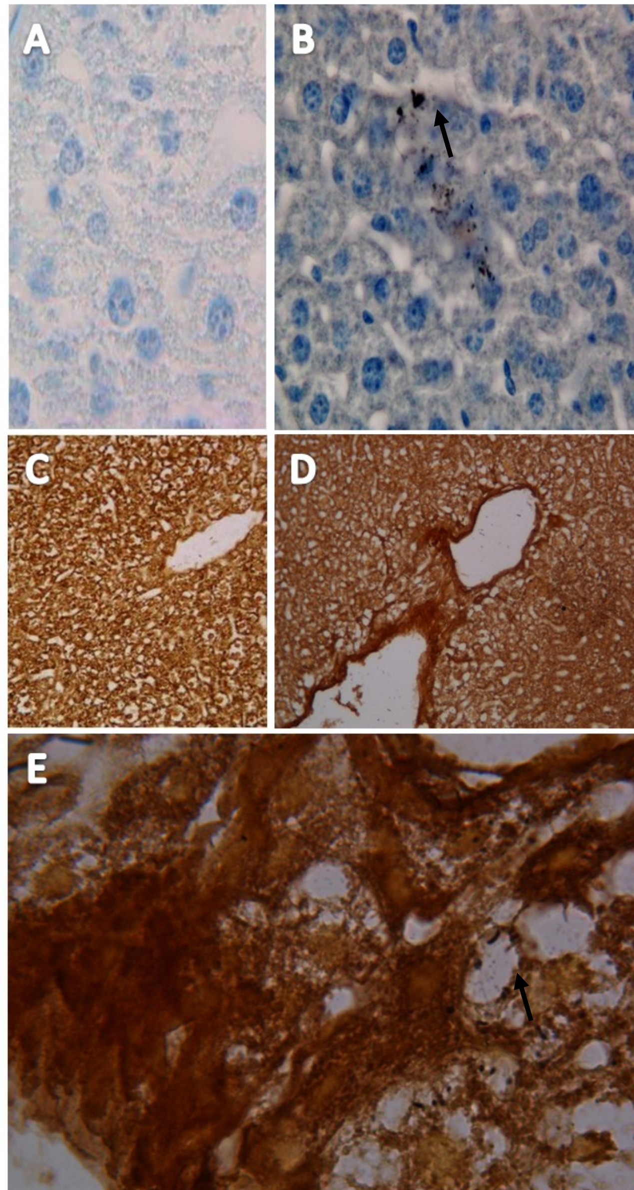

Figure 16. Immunohistochemistry analysis of liver samples of uninfected (A, x100 magnification) and *B. henselae* infected (B, x20 magnification) mice with an antibody against *B. henselae* showing the presence of organisms in infected samples. Warthin-Starry stain of uninfected (C, x20 magnification) and infected mice (D, x20 magnification and E, x100 magnification) showing the

presence of organisms surrounding the vessel and invading into the lumen of sinusoid (in collaboration with Dott.ssa G. Mansueto).

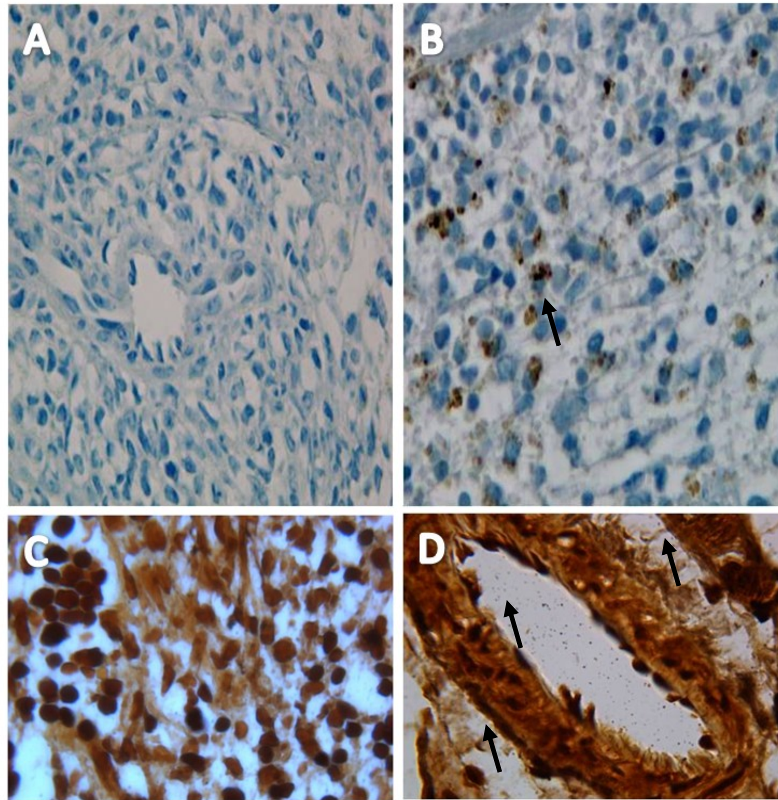

Figure 17. Immunohistochemistry analysis of spleen samples of uninfected (A, x100) and *B. henselae* infected mice (B, x100 magnification) with an antibody against *B. henselae.* Warthin-Starry stain of uninfected mice (C x100 magnification) and *B. henselae* infected (D x100 magnification) showing the presence of organisms present also in small aggregates (in collaboration with Dott.ssa G. Mansueto).

In order to confirm these results we have performed also a morphological analysis with blue of toluidine staining in liver and aorta of uninfected and *B. henselae* infected mice. We have found a normal portal triad in two groups of mice but in *B. henselae* infected mice a consistent collagen deposition was observed in the region surrounding the periportal and centrilobular veins (Figure 18). In the aorta of uninfected and *B. henselae* infected mice, we have observed a regular structure of the intima, the innermost layer, of the media, the middle layer with muscle and elastic fibers, and of the adventitia, the outer layer, that provides additional support and structure to the aorta. However, the intima layer of infected mice was resulted thicker than control mice (Figure 19).

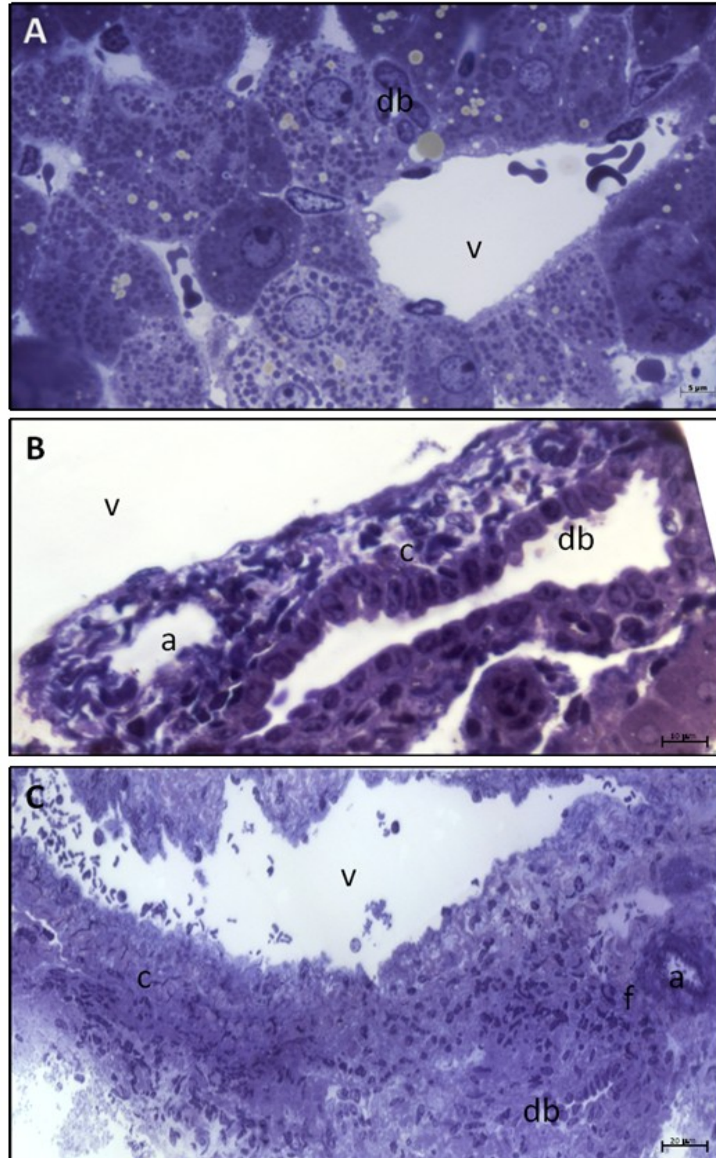

Figure 18. Figure 6. Section of the liver stained with toluidine blue of SCID mice uninfected (A) and *B. henselae* infected (B,C). (A) Regular hepatic portal vein (v), bile duct (db) and epithelial cells. (B, C) Regular structure of hepatic portal vein with hepatic artery (a), bile ductal (db) and portal vein but with consistent collagen (B, C) and presence of fibroblast (with Dott.ssa B. Avallone e Dr R. Cerciello).

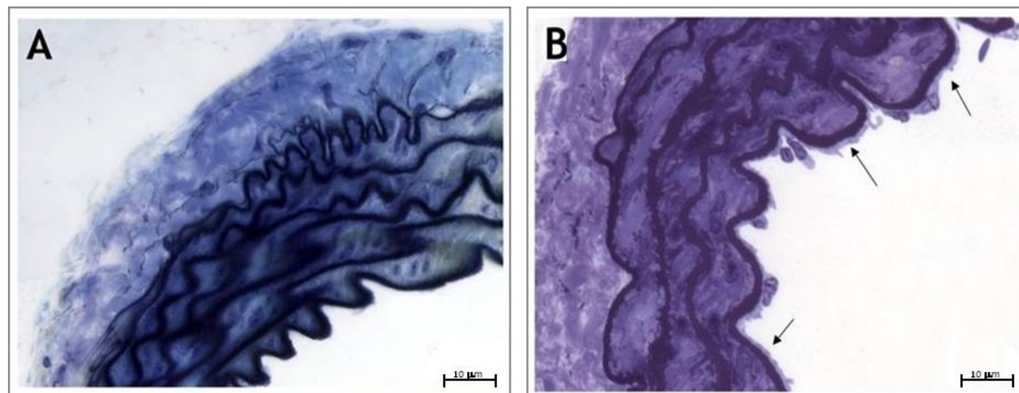

Figure 19. Section of the aorta stained with toluidine blue of SCID mice uninfected (A) and *B. henselae* infected (B). Normal aorta intima with endothelial cells, media with muscle and elastic fibres and adventitia with collagen (A). Intima layer appears thicker than control (arrow) while media and adventita layers appear regular (in collaboration with Dott.ssa B. Avallone e Dr R. Cerciello).

4.4.3 Ultrastructural analysis

The ultrastructural analysis with transmission electron microscopy performed on the liver of uninfected mice revealed a regular architecture of hepatic tissue with typical portal triad characterized by a small bile duct and portal vein with hepatocytes showing the polygonal shape, rounded nucleus with peripheral heterochromatin and massive presence of mitochondria and peroxisomes. The liver of *B. henselae* infected mice have shown macroareas with abundant collagen with the characteristic banding pattern under the endothelium of the portal vein with fibroblasts and myeloid cells. We have found also the presence of bacteria embedded in the collagen fibres and in space of Disse (Figure 20).

The same ultrastructural analysis were performed on aorta of uninfected and *B. henselae* infected mice. Aorta of all samples analyzed have shown any morphological alteration. In particular, in uninfected mice, the intima layer is regularly constituted by endothelial cells adherent to the basement membrane with elongated nucleus and peripheral heterochromatin. The tunica media constituted by muscle cells with elongated and longitudinal arrangement and with alternating elastic fibers. Finally, the adventitia composed of fibrocytes and collagen bundles with typical banding pattern (Figure 21). However, in *B. henselae* infected mice we have found that the layer of endothelium is thicker respect to control mice.

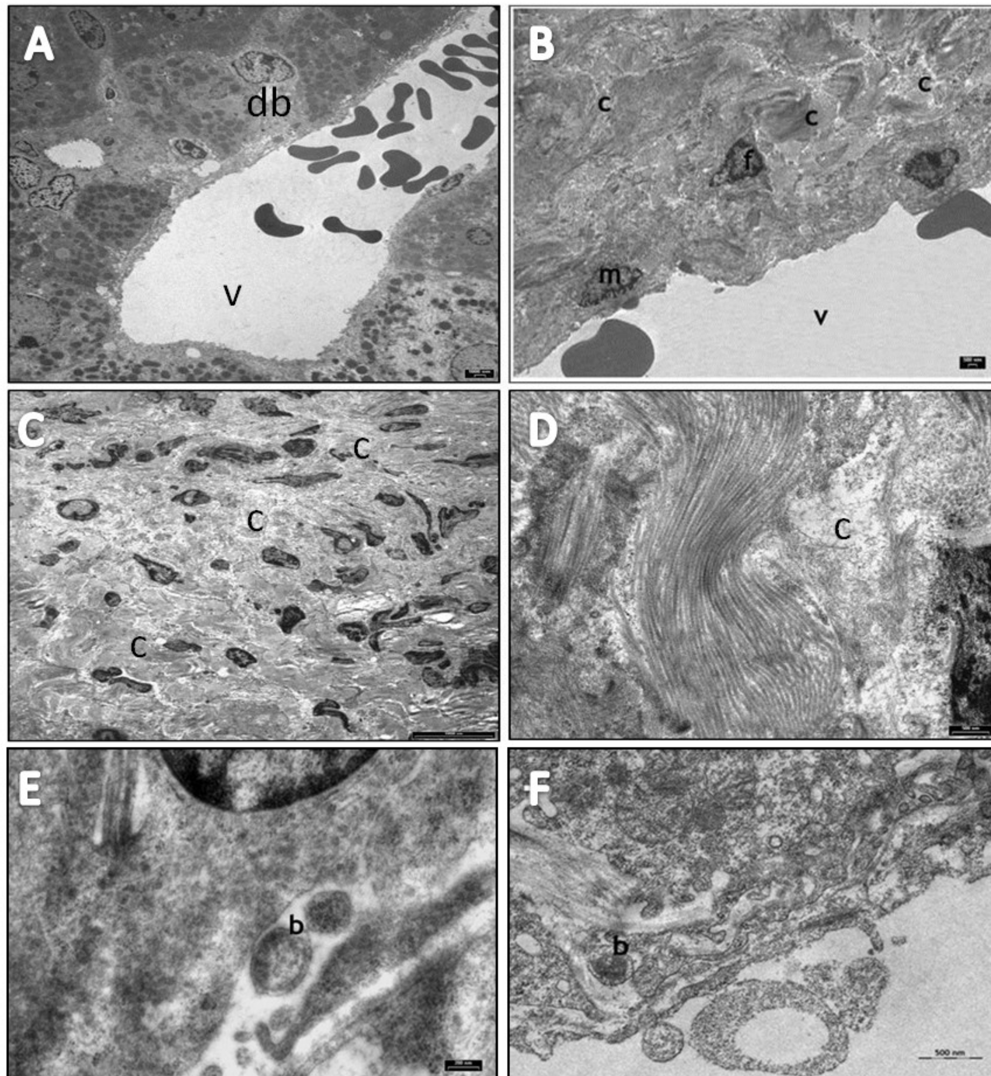

Figure 20. Ultrastructural analysis of liver of SCID mouse uninfected (A) and *B. henseale* infected (B-D). (A) portal triad characterized by a small bile duct (db) and portal vein (v) with hepatocytes with the characteristic polygonal shape, rounded nucleus with peripheral heterochromatin and massive presence of mitochondria and peroxisomes; (B) macroareas with abundant collagen under the endothelium of the portal vein (v) with fibroblasts (f) and myeloid cells (m); (C) macroareas with collagen (c) and fibroblasts with characteristic stellate shapes (f); (D). Magnification of collagen bundles (c) with the characteristic banding pattern. *B. henselae* bacterium embedded in area of collagen (E) and in the space of Disse (F) (in collaboration with Dott.ssa B. Avallone e Dr R. Cerciello).

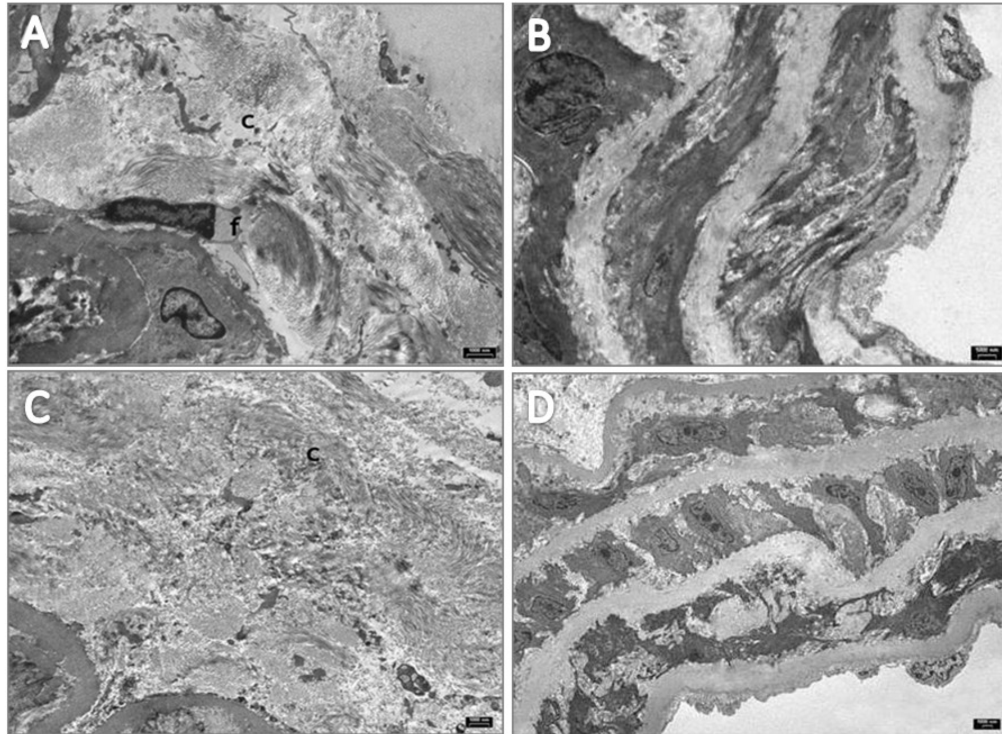

Figure 21. Ultrastructural analysis of the aorta of SCID mouse (A, B) control and infected with *B. henselae* (C, D). In both mice analyzed, the adventitia (A, C), the outer layer that provides additional support and structure to the aorta appears regular with fibroblasts (f) and collagen fibres (c). The intima of both uninfected and infected mice, the innermost layer of the vessel, appears regularly constituted by a monolayer of endothelial cells in direct contact with the blood and with a basal membrane. The tunica media is regular with smooth muscle cells and elastic fibers (B, D), but in *B. henselae* infected mice (D), the layer of endothelium is thicker respect to uninfected mice (in collaboration with Dott.ssa B. Avallone e Dr R. Cerciello).

4.4.4 EPC number in infected mice

After 36 days of infection, before the sacrifice, blood of mice was collected in order to evaluate whether the EPC number in immunocompromised mice was influenced by *B. henselae* infection. We have performed a cytofluorimetric assessment of EPC number using PE-anti-Sca-1 (phycoerythrin-conjugated stem cell antigen-1) and APC-anti-Flk-1 (allophycocyanin-conjugated fetal liver kinase-1) antibodies.
The mean number of EPCs were measured as cells double-positive for both Flk-1 and Sca-1 (Flk-1+/Sca-1+). As shown in Figure 22, the number of EPCs was found significantly increased in mice infected with *B. henselae* (1,4) compare to the uninfected group, whose the EPC number was considered as 100% (1.0).

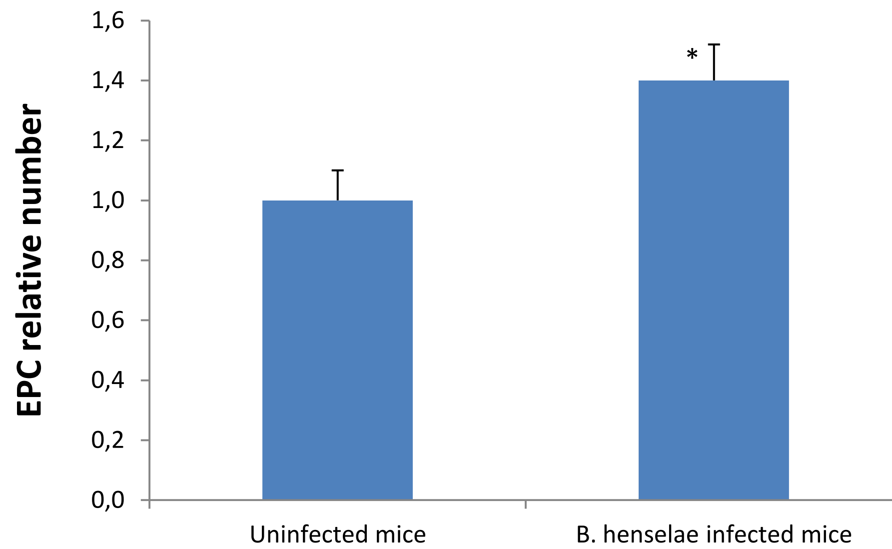

Figure 22. EPC number in uninfected and *B. henselae*-infected mice. EPC relative number of infected mice versus control was measured by FACS analysis. Sca-I/Flk-I double positive cells were considered as murine EPCs (*$p<0.05$).

5. Discussion

The results of our research established for the first time: a 21% of *B. henselae* seropositivity in patients awaiting HTx, an impairment of EPC number in *B. henselae*-infected patients and a correlation between *B. henselae* infection and the presence of HLA-B*35 allele in our patients. Furthermore, we have evaluated the tissue damage induced by *B. henselae* in a model of immunodeficient mice in which we also observed a significantly increase of the number of circulating EPCs.

The overall prevalence of *B. henselae* seropositivity was of 21% (n=8) in patients awaiting HTx (p=0.002). In particular, we have found that 8% of patients were positive for IgM (p=0.14) and 13% were positive for IgG antibodies (p=0.02). To our knowledge, this is the first report on the seroprevalence of *B. henselae* on patients awaiting HTx in our Region and in Italy. Our analysis supports the hypothesis that the age is a significant factor associated with prevalence of *B. henselae* infection in the patient population. Indeed, patients are older than controls and, likely they had more years of possible exposure to infective agents. However, in our population, this factor together with the state of general impairment of these patients due to the severe heart failure, likely increases the prevalence/incidence of infection. In post transplantation time, under the influence of immunosuppressive therapy these patients are at risk of developing infection diseases (Toyoda et al. 2013, Crudele et al. 2011). Despite significant advances in the management of solid organ transplantation, the infections still remain a considerable factor influencing transplantation outcome (Toyoda et al. 2013). Although in the post-transplantation period, many risk factors for infection are known, the epidemiology of infections in patients awaiting HTx and the effect of pre-transplantation infections in the post-transplantation period remain to be investigated (González-Padilla et al. 2013). Most of the guidelines for pre-transplantation management recommend the screening of patients potentially exposed to infectious agents, but they do not include opportunistic pathogens such as *B. henselae* (Fischer and Avery 2009). However, candidates awaiting HTx may have infections, caused by coagulase-negative staphylococci and aerobic gram-negative bacilli due to ventricular assist device implantations that are utilized as a bridge to transplantation or for the setting of congestive heart failure and general impairment.

Bartonella infections display different progression and organ involvement due to a balance between host and bacterial factors (Mogollon-Pasapera et al. 2009). Recently, several reports described cases of solid organ transplantation recipients who developed disseminated infections due to *B. henselae.* (Psarros et al. 2012, Rostad et al. 2012, Moulin et al. 2012). Cases of bartonellosis have been reported in liver, heart, kidney and lung transplantations and with a percentage of 28% of cases in subjects aged under 18 years (Poudel et al. 2014). Interestingly, the pathological lesions appear granulomatous as well as suppurative and the vasoproliferative disorders occur in lymph nodes, liver and

spleen. *B. henseale* infection in kidney recipients has been associated with acute rejection episode (Dharnidharka et al. 2002). The mechanism by which *B. henselae* causes vasoproliferation in immunocompromised hosts is still unclear, however, it has been demonstrated the ability of *B. henselae* to induce proliferation of endothelial cells. One hypothesis is that, in an immunocompromised host, the vasoproliferation bacterial-induced can overwhelm the granuloma formation, typical response of the immunocompetent individuals. The granuloma in immunocompetent subjects can limit the infection to the same lymph node region through a localized B-cell immune response regulated by dendritic cells that control the systemic spread of pathogens and represent a mechanism of effector phase of the immune response. In immunocompromised hosts, *Bartonella* bacteremia can occur followed by spreading of bacilli to liver, spleen, and cardiac valve triggering the vasoproliferative lesions as bacillary peliosis and/or bacillary angiomatosis (Vermi et al. 2006). Noteworthy, the most immunosuppressive drugs used in transplanted recipients, including cyclosporine and tacrolimus, target the cell-mediated immune response especially the T-cells leading to decrease the numbers of activated T cells. For this reason, the formation of granuloma that requires multiple factors such as T cells, might be impaired in the transplanted patients, thus promoting a switch versus vasoproliferative disorders (Rostad et al. 2012, Bonatti et al. 2006). However, at present there are no clear relashionship between the development of disseminated bartonellosis and any specific immnosuppressive regimen. In the transplanted patients, serological tests were useful diagnostic tool with a definition of a positive titre for IgM> 1:20 and IgG>1:64. PCR represents a diagnostic support that requires sample tissues but a negative result should not exclude the infection (Bonatti et al. 2006). Some studies support the possibility of *Bartonella* infections through blood transfusion, probably due to its intra-erythrocytic viability during the storage at 4°C (Ruiz et al. 2012). In subjects who develop bartonellosis few months after transplantation, a possible via of transmission of *B. henselae* could be also the organ donor (Scolfaro et al. 2008). Indeed, Scolfaro (2008) has reported a case of liver bartonellosis in a recipient whose donor was positive for *B. henselae* IgG antibodies (Scolfaro et al. 2008). Moreover, in immunocompromised patients a possible source of bartonellosis could be the reactivation of previous infections, although this complication is uncommon for bacteria (Psarros et al. 2012). At this regard, *B. henselae* may develop various processes to facilitate its uptake and permanence into intracellular compartment of the host cells, thus by promoting the reactivation (Eicher and Dehio 2012). Endothelial cells have been assumed as a potential niche *in vivo* of *B. henselae* and the location within these cells is also believed to protect this pathogen from antimicrobial agents (Harms and Dehio 2102). Inihibition of apoptosis, together with induction of cell proliferation, are pivotal mechanisms that allow the permanence of *B. henselae* into the host cells by contributing to vasoproliferative disorders (Deng et al. 2012; Harms and Dehio, 2012, Resto-Ruiz et al. 2002). When *B. henselae* gains access to the

human circulatory system, it disseminates from the point of inoculation and colonizes secondary foci showing preference for highly vascularized tissues like heart valves, liver and spleen (Chiaraviglio et al. 2010). Immediately after bacteria are released into the blood stream, where they efficiently infect erythrocytes and also reinfect the primary niche. Moreover, several studies have demonstrated that an alternative niche may be represented by HSC and EPCs (Mandle et al. 2005, Salvatore et al. 2008).

In our population study, we have found a reduced number of circulating EPCs in patients affected by advanced heart failure respect to a control population. Interestingly, the number of EPCs is further and significantly reduced in *B. henselae*-positive compared to *B. henselae*-negative patients. Obviously, our small clinical study cannot fully address the potential mechanisms underlying alteration of EPC number. However, alteration of number of EPCs has been described in a wide range of conditions such as cardiovascular, inflammatory, immune and infectious disease. Currently, EPC reduction is considered a risk factor that affects cardiovascular function and that promotes cardiovascular disease onset. Recently, it has been reported the ability of *B. henselae* to infect EPCs *in vitro* and *in vivo* (Salvatore et a. 2008, Sommese et al. 2012). Costa (2010) has observed a reduced number of EPCs in patients affected by Down Syndrome after *B. henselae* infection *in vitro*. The reduction of EPC number appears due to increased oxidative stress and up-regulation of angiogenesis and inflammation gene pathways (Costa et al. 2010). The hypothesis that infectious agents could impair the endothelial repair potential was reinforced by another study demonstrating an association between cerebral malaria and low levels of EPCs (Gyan et al. 2009). In patients awaiting HTx *B. henselae* could affect the proliferation of EPCs likely as result of increased metabolic demands of the host cells in order to cope with to bacterial infection.

Although several studies have highlighted the main pathogenic mechanisms of *B. henselae* infection, to date it is still unclear what is the primary niche of the bacterium *in vivo*. The results of our study and published data seem to support the hypothesis that EPCs could represent possible candidates for primary sites of infection. On this basis, mobilized EPCs could carry this pathogen to other organs and, more important, to the endothelium of microcirculation. It is plausible that the colonization of EPCs by *B. henselae* could profoundly alter the maintenance of vascular homeostatis by impairing the potential of these cells to regenerate the endothelial layer. Moreover, we can emphasize that the colonization of EPCs by *B. henselae* could play a pivotal role in the possible reactivation of bacterial infection in the post-transplantation when patients will be receiving immunosuppressive therapy. Therefore, the evaluation of EPCs by *B. henselae* can help you understand the pathogenic mechanisms underlying the infection as well as the mechanisms of vasoproliferative disorders occurring in immunocompromised individuals, but this issue remains to be elucidated. Therefore, in post-transplantation time, as a consequence of immunosuppressive therapy, the bacterial reactivation could result in a severe and disseminate bartonellosis that could compromise several organs. In

addition, *B. henselae* causes blood-culture negative endocarditis difficult to diagnose due to the negativity of the blood culture from native aortic valve tissue; this could be the reason why most patients show heart failure especially after valves implantation. Recently it has been reported that HTx may be considered as salvage treatment in selected patients with intractable infective endocarditis (Meininger et al. 2001, Edouard et al. 2015).
Therefore, we believe that an early diagnosis and an adequate treatment could be crucial in the pre-transplantation time in order to prevent the course of the disease in post-transplantation phase when the subjects will immunocompromised. Indeed, in patients IgG positive with current or past infection, the immunosuppressive therapy may exacerbate the bacterium that may persist in sanctuarie sites such as EPCs although patients received antibiotic therapy. Moreover, *B. henselae* can cause sustained infections in the blood stream for many months, perhaps partly due its association with cells of sanctuaries where likely there is a hospitable environment in which it is protected from the immune system.
In our study we have found a significant association between HLA-B*35 and *B. henselae* infection in patients awaiting HTx. It is noticeable that in this study the *B. henselae*-negative patients were selected as control in order to ensure a similar exposure to *B. henselae* between case and control groups. Our results imply for the first time that the susceptibility to *B. henselae* infection is significantly associated with the presence of HLA-B*35 allele. Several infectious diseases have been associated with specific HLA alleles and the mechanisms underlying this association not are always clear. Knowledge of the mechanisms of genetic protection or susceptibility to infectious diseases could contribute to understanding of pathogenic or protective mechanisms of these processes. Furthermore, some HLA molecules may be more or less efficient in the presentation of bacterial epitopes to T and B lymphocytes, by affecting the immune response and resulting in susceptibility to infection or protection from pathogens. Association between HLA and *B. henselae* infection has been studied only occasionally, finding the frequency of HLA-B*27 in patients with uveitis and serological characteristics of acute infection with *B. henselae* (Drancourt et al. 2004, Kerkhoff and Rothova 2000). However, it is unclear whether *B. henselae* infection modifies HLA-B*27 uveitis or HLA-B*27 positive patients are more likely to develop *B. henselae* infection. At this regard, the bacterial antigens can mimic HLA-B*27 antigen and be recognized as self. An association between HLA-B*35 and *Clamydia pneumonia* infection has been found in patients with coronary artery disease, showing that HLA-B*35 is a strongest-risk gene for infection (Palikhe et al. 2008). Moreover, the HLA-B*35 allele has been associated with chronic hepatitis B virus, hepatitis C virus and cytotoxic response against Aspergillus and Mycobacterium tubercolosis (Hammon et al. 2005, Kim et al. 2006). Several reports have showed a strong association between the presence of HLA-B*35 allele and the HIV infection. Indeed in several populations including Italian population, the presence of HLA-B*35 allele confers a high risk of HIV infection and it was

correlated with the disease progression (Kaur and Mehra 2009). Numerous reports suggest that HLA class I molecules due to their ability to present the antigens can act as signal transducing molecules able to influence individual reactivity to external stimuli. The presence of the HLA-B*35 allele has emerged as an important risk factor for the development of isolated pulmonary hypertension in Italian scleroderma patients in which HLA-B*35 may influence the production of endothelin-1, by promoting vasoconstriction, smooth muscle cell hypertrophy and intimal proliferation (Santaniello et al. 2006). The HLA-B*35 allele has been demonstrated to promote pathological cell activation and proliferation and gene transcription through the control of intracellular cation concentration in different cells (Lenna et al. 2010). It has been shown that *B. henselae*-infected host cells can induce proliferative and anti-apoptotic effects, through an intracellular Ca^{2+} rise from intracellular pools which leads to NFκB-directed pro-inflammatory activation and endothelial cell proliferation (McCord et al. 2007). We can hypothesized that the interaction between HLA-B*35 and *B. henselae* might influence the pathogenic mechanism triggered by this pathogen and HLA-B*35 could represent in our population a genetic marker of *B. henselae* infection.

Although performed on a small number of patients enrolled in waiting list for HTx in our region, our study indicated that HLA-B*35 confers a *B. henselae*-related risk to patients with heart failure. However, a better design and more patients will be needed to confirm these results in order to understand whether this association depend on a different ability of HLA molecules to present bacterial antigens to T cells or if HLA-B*35 affects the signals transduced into the cells such as those proliferative or apoptotic. Therefore, the different HLA alleles would be important factors influencing the immune responses against *B. henselae* and the outcome of the disease that should be taken into account in cellular-immune response studies.

The use of systems *in vitro* of *B. henselae* infection have allowed to understand many of the mechanisms of *Bartonella*-host interaction at cellular and molecular level including the ability of this pathogen to induce endothelial cell proliferation. However, the ability of *B. henselae* to support a chronic infection and to produce its various pathological features in immunocompetent or immunocompromised hosts can be well understand only through an *in vivo* model. In a previous study, Sommese (2012) has demonstrated the tissue damage induced by *B. henselae* in a model of immunocompetent mice reporting also the internalization and colocalization of bacterium in several tissues. Several alterations of liver and aorta of animal infected with *B. henselae* were demonstrated compared to uninfected controls. In this system, the damage induced by *B. henselae* in liver and aorta can be prevented by the coinfection with *B. fragilis* through the PSA. The authors, for the first time have observed a dramatic reduction of EPCs in *B. henselae*-infected mice while the coinfection with *B. fragilis* has been able to restore the EPC number (Sommese et al. 2012).

In our study, we have developed a model of *B. henselae* infection represented by immunocompromised SCID\Beige mice deficient in B, T and natural killer cells in order to provide an experimental model for human disease in immunocompromised host like the transplanted patients undergo to immunosuppressive therapy. Our morphological and ultrastructural analysis performed on SCID mice infected with *B. henselae* showed that these mice tolerate infection at high concentrations as well as the human immunocompromised that support chronic bloostream *B. henselae* infection, with the risk of endovascular infections. Surprisingly the tolerance manifested by mice after 36 days of infection appears to contrast with what would be expected during a bacteremia with other Gram-negative pathogens where death would follow the infection without a rapid antibiotic therapy.
In the tissue of *B. henselae*-infected mice, we have found a significant collagen deposition, this feature was also found in human disease such as bacillary angiomatosis and peliosis hepatis in which much attention is focused also on proliferating cell component of the response of the host (Cockerell et al. 1991; Borczuk et al. 1998). However, in bacillary angiomatosis, proliferating capillary structures have been shown embedded in an extracellular matrix composed by bacteria, immune cells and fibrous material (Cockerell et al. 1991; Borczuk et al. 1998). In our study, immunohystochemistry and the Warthin-Starry stain used for identification of *B. henselae* in tissue section showed the presence of bacteria in the lesional areas in which they appear in small and large clusters and individually. This result has been confirmed by transmission electronic microscopy analysis that revealed the presence of bacteria in the collagen fibres and in space of Disse but not within endothelial or other cell types.
Taking together, these observations suggest that the deposition of collagen can be a general pathological response to *B. henselae* infection, evident both in the tissue of immunocompromised mice models and in human lesions of bacillary angiomatosis and peliosis. It is possible speculate that the deposition of collagen allows the host to contain the pathogen but this way offers to the bacterium an environment where it is protected from the immune response. It is known that *in vitro, B. henselae* adheres to the collagen fibers through the surface adhesion molecules, this process could promote the beneficial association with fibers of collagen as observed *in vivo* (Dabo et al. 2006). The generalized collagen deposition, observed in our animal model, could be considered as a failed attempt to develop a granuloma, in an immunocompromised model, likely related to the time compared to infection in which the observation was performed. Several studies *in vitro* have reported the evidence of intracellular replication of *B. henselae* in erythroid and endothelial cells (Mandle et al. 2005, Kempf et al. 2001). In the *in vivo* model we not have observed the intracellular presence of the bacteria. Likely, *B. henselae* can replicate only in specific vascular areas that we have not evaluated, or the *in vitro* system could not reproduce the mechanisms of infection *in vivo*. Furthermore, we not have observed in immunocompromised

murine model the typical endothelial proliferation. Interestingly, we have found a significant increase of EPC number in *B. henselae* infected mice respect to control. Several stimuli can induce a mobilization of EPCs from bone marrow and their migration at the site of damage including HIF-1-mediated Stromal cell-Derived Factor-1 (SDF-1) and VEGF, these two signaling proteins bind their respective receptors on EPCs by promoting the recruitment in the site of angio- and vasculogenesis (Napoli et al. 2011). The angiogenic reprogramming of host cells infected *in vitro* with *B. henselae* seems to be regulated by the activation of HIF-1 that stimulates angiogenesis and secretion of VEGF. The secretion of HIF-1 and VEGF have been demonstrated in patients with bacillary angiomatosis and *in vitro* system (Kempf et al. 2005 and Riess et al. 2004). We can hypothesize that the increased number of EPCs observed in immunocompromised mice infected with *B. henselae* could be related to the onset of bacillary angiomatosis typical of immunocompromised subjects in which, the vasoproliferative process may require a great supply of endothelial precursors (Figure 23). We suppose that the lack of endothelial proliferation could be related to the time when we have performed our analysis. Furthermore, the increased EPC number in immunocompromised *B. henselae* infected mice could also represent a defense mechanism used by the host, considering the macrophagic features of the EPCs. Indeed, early-EPCs derive from a population of CD14+ cells, and they are able to secrete angiogenic growth factors, thus can stimulate angiogenesis in a paracrine manner.
However, further investigations should also focus on the course of infection in immunocompromised mice because *B. henselae* infection causes vasculoproliferative disease only in immunocompromised subjects and the involvement of EPCs in this process needs to be better understand. Interestingly, EPCs could represent a primary niche of *B. henselae* that can promote the bacterial reactivation in immunosuppression conditions but they could be also involved in the vasoproliferative disorders occurring in the immunodeficient subjects including transplanted recipients. The SCID\Beige mouse model used should provide an experimental basis for progress of understanding the pathogenesis of *B. henselae* infection and the immune response to these infections.

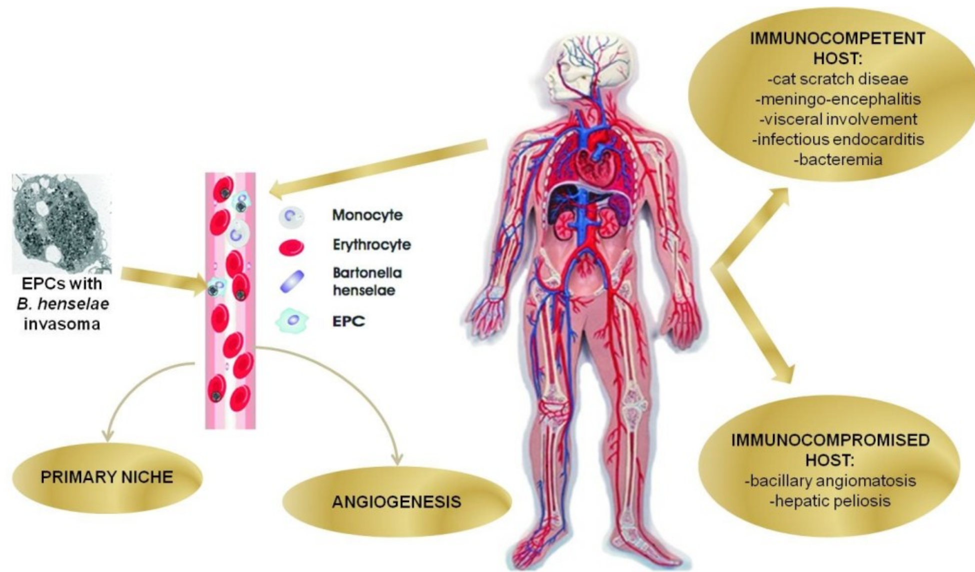

Figure 23. *B. henselae* and EPCs. The EPCs can internalize *B. henselae*, thus by establishing a circulating niche of this pathogen and by promoting vascular angiogenesis.

6. Conclusions

We have demonstrated a high prevalence of *B. henselae* infection in patients awaiting HTx enrolled in our Regional Reference list for organ transplantation. We have found a significant correlation between the *B. henselae* infection and the presence of HLA-B*35 allele together with a significant impairment of number in circulating EPCs in patients with advanced heart failure. Our retrospective observational study, although performed on a small number of patients only in pre-transplantation time indicates that *B. henselae*, together with other emerging bacteria, should be included as a routine analysis in the list of opportunistic infections. Therefore, the pre-transplantation monitoring of *B. henselae* infections may be useful in the management of patients in post-transplantation phase when these patients will undergo to immunosuppressive therapy. The mechanisms underlying the reduction in the number of circulating EPCs (that represent an *in vivo* niche of *B. henselae*) might promote the bacterial reactivation. The pathophysiological consequences of these events in patients with heart failure as well as the HLA association need further investigations.

In addition, we have evaluated the tissue damage in a model of immunocompromised mice by revealing a strong deposition of collagen after *B. henselae* infection and an increase of EPC number that suggest an involvement of these cells in the immune response of mice infected but also in the triggering the vasoproliferative process. However, further investigations should be focused on the course of infection in our patients both in the post transplantation time as well as in the animal model in order to understand the pathogenic mechanism of *B. henselae*.

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