Human complement receptor type 1 (CR1) protein levels and genetic variants in chronic Chagas Disease

Thaisa Lucas Sandri1,2, Kárita Cláudia Freitas Lidani3, Fabiana Antunes Andrade3, Christian G. Meyer2,3,4, Peter G. Kremsner2, Iara J. de Messias-Reason1 & Thirumalaisamy P. Velavan2,3,4

Complement is an essential element in both innate and acquired immunity contributing to the immunopathogenesis of many disorders, including Chagas Disease (CD). Human complement receptor 1 (CR1) plays a role in the clearance of complement opsonized molecules and may facilitate the entry of pathogens into host cells. Distinct CR1 exon 29 variants have been found associated with CR1 expression levels, increased susceptibility and pathophysiology of several diseases. In this study, CR1 plasma levels were assessed by ELISA and CR1 variants in exon 29 by sequencing in a Brazilian cohort of 232 chronic CD patients and 104 healthy controls. CR1 levels were significantly decreased in CD patients compared to controls ($p < 0.0001$). The CR1 rs1704660G, rs17047661G and rs6691117G variants were significantly associated with CD and in high linkage disequilibrium. The CR1*AGAGTG haplotype was associated with $T. cruzi$ infection ($p = 0.035$, OR 3.99, CI 1.1-14.15) whereas CR1*AGGCTG was related to the risk of chagasic cardiomyopathy ($p = 0.028$, OR 12.15, CI 1.13-113). This is the first study that provides insights on the role of CR1 in development and clinical presentation of chronic CD.
role in the infection process, while the parasite deactivates the lectin complement pathway, which ultimately could favor *T. cruzi* cell internalization mediated by receptors for both molecules, including CR1.

The complement system is essential in both innate and acquired immunity, contributing to the immunopathogenesis of a variety of diseases, including CD. CR1, or CD35, is a multi-functional polymorphic glycoprotein, which occurs as a soluble or transmembrane protein expressed on peripheral blood cells including monocytes and erythrocytes, natural killer cells as well as on B and T cells. CR1 is known to enhance phagocytosis of particles opsonized with C3b, C4b, C1q, MBL, and ficolin-2 as well as to facilitate the clearance of immune complexes by binding to CR1 on erythrocytes and macrophages for further disposal. The *CR1* gene is located on chromosome 1q32.2 (OMIM 120620) and belongs to the Regulator of Complement Activation family, which is characterized by small consensus repeats, also known as complement control protein repeats. Genetic variability may influence CR1 expression including its molecular weight and the density of CR1 molecules on cell surfaces.

It has been demonstrated that CR1 is involved in the pathogenesis of several infectious diseases either by facilitating pathogens entry into host cells in some cases or by down-modulating complement activation in others. CR1 was shown to mediate immune opsonization of *Leishmania* amastigotes and promastigotes, *Plasmodium falciparum*, *Mycobacterium tuberculosis*, *M. leprae*, HIV, SARS-CoV, adenovirus, hepatitis C virus, and West Nile Virus.

Besides the role of CR1 in facilitating the entry of intracellular pathogens into host cells, CR1 protein levels were shown to be associated with the pathogenesis of different diseases including malaria, tuberculosis, lepromatous leprosy, severe acute respiratory syndrome, HIV infection among others. The *CR1* genetic variants in exon 29 evaluated in this study (rs17047660, rs17047661, rs4844609 and rs6691117) are of particular interest since all are non-synonymous variants that are situated at the binding site for C1q, ficolins and MBL having thereby potential to influence the complement induced phagocytosis. The present study aimed to assess if the genetic variants in exon 29 and CR1 levels are associated with development and clinical presentation of chronic CD.

### Results

**CR1 plasma levels.** CR1 plasma levels were significantly lower in CD patients compared to controls (*p* < 0.0001), (Fig. 1). When comparing controls to each clinical form separately, statistical differences were also observed for CR1 levels between controls and the indeterminate form (*p* = 0.0002), cardiac form (*p* < 0.0001), digestive form (*p* < 0.0001), and cardiodigestive form (*p* < 0.0001) (Fig. 1). Comparison of CR1 levels between asymptomatic (indeterminate form) and symptomatic patients showed no statistical difference.

**Association of CR1 variants with Chagas disease.** The distribution of *CR1* genotypes in controls was in Hardy-Weinberg equilibrium (*p* > 0.05), in patients with chronic CD three SNPs (rs17047660, rs17047661, rs4844609) were not in HW equilibrium, which may be due to disease association. The frequencies of *CR1* variants rs17047660G (*p* = 0.02, OR 5.06, 95%CI 1.17-21.81), rs17047661G (*p* = 0.0042, OR 3.03, 95%CI 1.34-9.9) and rs6691117G (*p* = 0.015, OR 1.6, 95%CI 1.09-2.35) were significantly higher in CD patients compared to controls (Table 1). Also, the frequencies of the *CR1* genotypes rs17047661AG and rs17047661GG (*p* = 0.015, OR 3.0, 95%CI 1.25-7.49) and rs6691117AG and rs6691117GG (*p* = 0.004, OR 2.2, 95%CI 1.26-3.53) were significantly higher in chronic CD patients than in controls (Table 1).

When analyzing CD patients according to their clinical presentation in relation to controls, the rs6691117G allele occurred more frequently among asymptomatic indeterminate form of CD (*p* = 0.02, OR 1.7, 95%CI 1.09-2.69) and in patients presenting with the digestive form of CD (*p* = 0.025, OR 2.4, 95%CI 1.18-5.0) (Table 1). In addition, carriers of the G allele (rs6691117AG and rs6691117GG) were rather present among asymptomatic patients than in controls (*p* = 0.006, OR 2.3, 95%CI 1.28-4.27) (Table 1). A significant association with the cardiac
| CRI genetic variants | Control n = 102 (%) | CD Patients n = 220 (%) | Indeterminate n = 87 (%) | Cardiac n = 77 (%) | Digestive n = 19 (%) | Cardio digestive n = 31 (%) | CD Patients vs. Controls p value; OR [95% CI] | Indeterminate vs. Controls p value; OR [95% CI] | Cardiac vs. Controls p value; OR [95% CI] | Digestive vs. Controls p value; OR [95% CI] |
|---------------------|---------------------|-------------------------|-------------------------|-------------------|-------------------|---------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| rs17259045A/G | AA 82 (80) | 190 (87) | 75 (86) | 69 (90) | 16 (84) | 23 (81) | NS | NS | NS | NS |
| | AG 20 (20) | 27 (12) | 11 (13) | 7 (9) | 3 (16) | 5 (16) | | | | |
| | GG 0 | 3 (1) | 1 (1) | 1 (1) | 0 | 1 (3) | | | | |
| | A* 184 (90) | 407 (92) | 161 (92) | 145 (94) | 35 (92) | 55 (89) | NS | NS | NS | NS |
| | G 20 (10) | 33 (8) | 13 (8) | 9 (6) | 3 (8) | 7 (11) | | | | |
| rs41274768G/A | GG 98 (96) | 198 (90) | 80 (92) | 70 (91) | 16 (84) | 27 (87) | | | | |
| | GA 4 (4) | 22 (10) | 7 (8) | 7 (9) | 3 (16) | 4 (13) | | | | |
| | AA 0 | 0 | 0 | 0 | 0 | 0 | | | | |
| | G* 200 (98) | 418 (95) | 167 (96) | 147 (95) | 35 (92) | 58 (93) | NS | NS | NS | NS |
| | A 4 (2) | 22 (3) | 7 (4) | 7 (5) | 3 (8) | 4 (7) | | | | |
| rs17047660A/G | AA 100 (98) | 202 (92) | 81 (93) | 70 (91) | 19 (100) | 28 (90) | | | | |
| | AG 2 (2) | 15 (7) | 5 (6) | 6 (8) | 0 | 2 (6) | | | | |
| | GG 0 | 3 (1) | 1 (1) | 1 (1) | 0 | 1 (3) | | | | |
| | A* 202 (99) | 419 (95) | 167 (96) | 146 (95) | 38 (100) | 58 (94) | | | | |
| | G 2 (1) | 21 (5) | 7 (4) | 8 (5) | 0 | 4 (6) | | | | |
| rs17047661A/G | AA 95 (93) | 183 (83) | 76 (87) | 61 (79) | 16 (84) | 27 (87) | | | | |
| | AG 7 (7) | 31 (14) | 9 (10) | 13 (17) | 3 (16) | 3 (10) | | | | |
| | GG 0 | 6 (3) | 2 (2) | 3 (4) | 0 | 1 (3) | | | | |
| | A* 197 (97) | 397 (90) | 161 (93) | 135 (88) | 35 (92) | 57 (92) | | | | |
| | G 7 (2) | 43 (10) | 13 (7) | 19 (12) | 3 (8) | 5 (8) | | | | |
| rs4844609T/A | TT 100 (98) | 213 (97) | 84 (97) | 75 (97) | 18 (95) | 30 (97) | | | | |
| | TA 2 (2) | 4 (2) | 3 (3) | 1 (1.5) | 0 | 0 | | | | |
| | AA 0 | 3 (1) | 0 | 1 (1.5) | 1 (5) | 1 (3) | | | | |
| | T* 202 (99) | 430 (98) | 171 (98) | 151 (98) | 36 (95) | 60 (97) | | | | |
| | G 2 (1) | 10 (2) | 3 (2) | 3 (2) | 2 (5) | 2 (3) | | | | |
| rs6691117A/G | AA 62 (61) | 99 (45) | 36 (41) | 39 (51) | 7 (37) | 16 (52) | | | | |
| | AG 33 (32) | 99 (45) | 43 (49) | 31 (40) | 8 (42) | 12 (39) | | | | |
| | GG 7 (7) | 22 (10) | 8 (9) | 7 (9) | 4 (21) | 3 (10) | | | | |
| | A* 157 (77) | 287 (68) | 115 (66) | 109 (71) | 22 (58) | 44 (71) | | | | |
| | G 47 (23) | 143 (32) | 59 (34) | 45 (29) | 16 (42) | 18 (29) | | | | |

Table 1. CRI genotypes and allele frequencies in patients with chronic CD and healthy controls. NA: Not applicable, NS: Not significant, *Major allele in the investigated population.

A total of 15 CRI haplotypes were observed, they were reconstructed from the six CRI variants (rs17259045, rs41274768, rs17047660, rs17047661, rs4844609, rs6691117) investigated in the study (Fig. 2). The frequency of CRI*AGAGTG haplotype was significantly increased among CD patients (p = 0.035, OR 3.9, 95%CI 1.10-14.15), in patients with cardiomyopathy without ECHO alteration (p = 0.03, OR 5.5, 95%CI 1.17-25.8), and in cardiomyopathy patients without heart failure (p = 0.005, OR 7.7, 95%CI 1.84-32.7) than among controls. In addition, CRI*AGGGTG was significantly associated with cardiomyopathy (p = 0.028, OR 12.1, 95%CI 1.3-113) and with the absence of heart failure (p = 0.037, OR 11.1, 95%CI 1.15-107) in comparison to controls (Table 3). Linkage disequilibrium (LD) patterns of the CRI variants are given in Fig. 3. Strong LD was observed only in chronic CD patients. The LD plot indicates that rs17259045, rs41274768, rs17047660, and rs17047661 were in strong LD with rs6691117; therefore, rs17047660 was also in strong LD with rs17047661.
| CRI genetic variants | Control\(n=102\) (%) | Indeterminate\(n=87\) (%) | Without ECHO alteration\(n=24\) (%) | With ECHO alteration\(n=74\) (%) | Without Heart Failure\(n=54\) (%) | Heart Failure vs. Indeterminate p value OR [95%CI] | Without ECHO alteration vs. Indeterminate p value OR [95%CI] | Without Heart Failure vs. Indeterminate p value OR [95%CI] | Heart Failure vs. Without Heart Failure p value OR [95%CI] |
|---------------------|----------------------|--------------------------|----------------------------------|----------------------------------|----------------------------------|-----------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|
| rs17259045A/G       | AA 82 (80)           | 75 (86)                  | 21 (88)                           | 63 (85)                          | 45 (88)                          | 39 (83)                                 | NS                                        | NS                                        | NS                                        |
|                     | AG 20 (20)           | 11 (13)                  | 1 (4)                             | 11 (15)                          | 4 (8)                            | 8 (17)                                  | NS                                        | NS                                        | NS                                        |
|                     | GG 0                 | 1 (1)                    | 2 (8)                             | 0                               | 2 (4)                            | 0                                       | NS                                        | NS                                        | NS                                        |
| A*                  | 184 (90)             | 161 (92)                 | 43 (90)                           | 137 (93)                         | 94 (92)                          | 86 (91)                                 | NS                                        | NS                                        | NS                                        |
| G                   | 20 (10)              | 13 (8)                   | 5 (10)                            | 11 (7)                           | 8 (8)                            | 8 (9)                                   | NS                                        | NS                                        | NS                                        |
| rs41274768G/A       | GG 98 (96)           | 80 (92)                  | 23 (96)                           | 66 (89)                          | 45 (88)                          | 44 (94)                                 | NS                                        | NS                                        | NS                                        |
|                     | GA 4 (4)             | 7 (8)                    | 1 (4)                             | 8 (11)                           | 6 (12)                           | 3 (6)                                   | NS                                        | NS                                        | NS                                        |
|                     | AA 0                 | 0                       | 0                                 | 0                               | 0                               | 0                                       | NS                                        | NS                                        | NS                                        |
| A*                  | 200 (98)             | 167 (96)                 | 47 (98)                           | 140 (95)                         | 96 (94)                          | 91 (97)                                 | NS                                        | NS                                        | NS                                        |
| G                   | 4 (2)                | 7 (4)                    | 1 (2)                             | 8 (5)                            | 6 (6)                            | 3 (3)                                   | NS                                        | NS                                        | NS                                        |
| rs1047660A/G        | AA 100 (98)          | 81 (93)                  | 21 (88)                           | 67 (91)                          | 44 (86)                          | 44 (94)                                 | NS                                        | NS                                        | NS                                        |
|                     | AG 2 (2)             | 5 (6)                    | 3 (13)                            | 5 (7)                            | 7 (14)                           | 1 (2)                                   | NS                                        | NS                                        | NS                                        |
|                     | GG 0                 | 1 (1)                    | 0                                 | 2 (3)                            | 0                               | 2 (4)                                   | NS                                        | NS                                        | NS                                        |
| A*                  | 202 (99)             | 167 (96)                 | 45 (94)                           | 139 (94)                         | 95 (93)                          | 89 (95)                                 | NS                                        | NS                                        | NS                                        |
| G                   | 2 (1)                | 7 (4)                    | 3 (6)                             | 9 (6)                            | 7 (7)                            | 5 (5)                                   | NS                                        | NS                                        | NS                                        |
| rs17047661A/G       | AA 95 (93)           | 76 (87)                  | 16 (69)                           | 63 (85)                          | 35 (69)                          | 44 (93)                                 | NS                                        | NS                                        | NS                                        |
|                     | AG 7 (7)             | 9 (10)                   | 7 (25)                            | 8 (11)                           | 13 (25)                          | 2 (4)                                   | NS                                        | NS                                        | NS                                        |
|                     | GG 0                 | 2 (2)                    | 1 (6)                             | 3 (4)                            | 3 (6)                            | 1 (2)                                   | NS                                        | NS                                        | NS                                        |
| A*                  | 197 (97)             | 161 (93)                 | 39 (81)                           | 134 (91)                         | 83 (81)                          | 90 (94)                                 | NS                                        | NS                                        | NS                                        |
| G                   | 7 (2)                | 13 (7)                   | 9 (19)                            | 14 (9)                           | 19 (19)                          | 4 (4)                                   | NS                                        | NS                                        | NS                                        |
| rs4446097/A         | TT 100 (98)          | 84 (97)                  | 24 (100)                          | 71 (96)                          | 51 (100)                         | 44 (94)                                 | NS                                        | NS                                        | NS                                        |
|                     | TA 2 (2)             | 3 (3)                    | 0                                 | 3 (1)                            | 0                               | 1 (2)                                   | NA                                        | NA                                        | NA                                        |
|                     | AA 0                 | 0                       | 0                                 | 2 (3)                            | 0                               | 2 (4)                                   | NA                                        | NA                                        | NA                                        |
| T*                  | 202 (99)             | 171 (98)                 | 48 (100)                          | 143 (97)                         | 102 (100)                        | 89 (95)                                 | NS                                        | NS                                        | NS                                        |
| A                   | 2 (1)                | 3 (2)                    | 0                                 | 5 (3)                            | 0                               | 5 (5)                                   | NA                                        | NA                                        | NA                                        |
| rs6691117A/G        | AA 62 (61)           | 36 (41)                  | 12 (50)                           | 40 (54)                          | 24 (47)                          | 28 (60)                                 | NS                                        | NS                                        | NS                                        |
|                     | AG 33 (32)           | 43 (49)                  | 9 (37)                            | 28 (38)                          | 20 (39)                          | 17 (36)                                 | NS                                        | NS                                        | NS                                        |
|                     | GG 7 (7)             | 8 (9)                    | 3 (13)                            | 6 (8)                            | 7 (14)                           | 2 (4)                                   | NS                                        | NS                                        | NS                                        |
| A*                  | 157 (77)             | 115 (66)                 | 33 (69)                           | 108 (73)                         | 68 (67)                          | 73 (78)                                 | NS                                        | NS                                        | NS                                        |
| G                   | 47 (23)              | 59 (34)                  | 15 (31)                           | 40 (27)                          | 34 (33)                          | 21 (22)                                 | NS                                        | NS                                        | NS                                        |

Table 2. CRI genotypes and allele frequencies in patients with chronic CD based on cardiac impairment. NA: Not applicable, NS: Not significant, *Major allele in the investigated population.

Discussion
In order to maintain its life cycle after transmission by triatomine vectors to a human host, *T. cruzi* needs to evade host immune attack and develops further intracellularly. The successful entrance of *T. cruzi* into host cells depends on the down-regulation of complement activation by parasite regulatory molecules and by its binding to complement receptors such as CR1. Thus, complement system and CR1 have an important role both in the establishment of *T. cruzi* infection and sustenance of the chronic phase. In this study, the CRI genetic variants in exon 29 were investigated in patients with chronic CD in order to assess their role in the modulation of CR1 levels as well as in the development and in the clinical progression of the disease.

Patients with chronic CD had significantly decreased levels of CR1 compared to healthy controls. Plasma levels observed in the control group were in accordance with those reported in other studies. The reduced CR1 expression on erythrocytes combined with increased levels of immune complexes has been demonstrated in the pathogenesis of HIV, SARS-CoV, *M. tuberculosis* and *M. leprae* infections. In leprosy, AIDS, and tuberculosis, the reduction of CR1 levels is disease regulated, demonstrating that this condition is acquired rather than inherited. Moreover, it is known that, similarly to mechanisms used by other pathogens, *T. cruzi* uses C1q to promote C1-dependent phagocytosis as well as MBL and ficolin-2 to promote opsonization via CR1 as a strategy to evade the host immune system and infect host cells.

Interestingly, patients with cardiomyopathy had lower CR1 plasma levels than asymptomatic patients, which might indicate either consumption due to increased complement activation or lower production associated with this clinical manifestation. In fact, chagasic cardiomyopathy is known to be associated with inflammatory process and tissue damage, as observed in various inflammatory and infectious conditions. Moreover, one of the consequences of the persistent myocardial damage in CD is left ventricular dilation with systolic dysfunction. For this reason, left ventricular systolic function was evaluated in CD patients using the Left Ventricular Ejection Fraction (LVEF). Despite the important role of the complement system in cardiovascular diseases...
such as atherosclerosis, myocardial infarction, and acute ischaemic stroke, no correlation between CR1 levels and LVEF was found. This finding corroborates with data from a study on patients with acute myocardial infarction.

It is known that CR1 levels may be influenced by infections and that their expression is associated with genetic as well as acquired factors. It was observed in this study that lower levels of CR1 were associated with rs6691117 GG genotype in the controls, but not in patients. Two other studies found this genotype associated with lower erythrocyte sedimentation rate and with preterm birth. These findings indicate that rs6691117 GG genotype may modulate CR1 expression. Since there was no association between the rs6691117 GG and CR1 levels in the patients, the reduction of CR1 levels in chronic CD is probably due to the disease process. An anti-inflammatory role for CR1 was already observed in experimental studies where CR1 was able to prevent tissue injury induced by complement activation. Considering that chronic CD is associated with inflammation, it is possible that the low levels of CR1 in CD patients may be related to its anti-inflammatory effect and consumption due to complement activation. However, the exact mechanism, which controls the expression of CR1 in CD patients is still unclear.

The positive association of AG and GG genotypes (in variants rs17047661, rs6691117) and the G allele (in variant rs17047660) observed with chronic CD may be related to the functional properties of the CR1 molecule. These variants lead to the substitutions of amino acids in the CR1 molecule which may affect the folding and the affinity of CR1 to C3b, C4b and C1q/MBL/ficolin. The allele rs6691117G was also related to a low ratio of CR1 expression in erythrocyte membranes. In addition, the alleles rs17047660G and rs17047661G were previously associated with severe malaria, sickle cell anemia, and showed to have protective effects against M. leprae and M. tuberculosis infection, while allele rs6691117G increased risk of Alzheimer disease, gastric cancer, non-small cell lung cancer and preterm birth.

Moreover, the allele rs17047661G and CR1*AGGGTG and AGAGTG haplotypes were related to early stages of CD cardiac form indicating that these variants might predispose to clinical progression of chronic patients with CD. Since the pathogenesis of CCC involves parasite persistence in different tissues as well as continuous low-grade parasitemia, inflammatory process and immune mediated-myocardial injury, it is possible that protein products of these CR1 variants may augment T. cruzi binding with consequent cellular internalization besides having an immunomodulatory effect.

A limitation of the present study is the lack of baseline CR1 plasma levels in patients with acute CD. Acute CD patients are difficult to diagnose clinically and hence the measurement of CR1 levels was not possible during their early stages of infection that might serve as a baseline measurement. The Ambulatory of Chagas Disease of Hospital das Clínicas (Federal University of Paraná) enrolls only chronic CD patients, thus making the access to acute CD patients impossible.

In conclusion, this study reports that CR1 variants are associated with the risk of T. cruzi infection and to progression to chagasic cardiomyopathy. Besides that, the low of CR1 levels observed in CD patients is possibly due to the disease process. This is the first study that provides insights on the role of CR1 in development and clinical presentation of chronic CD. Nevertheless, further studies are necessary to confirm these findings.
Methods

Study Population. A total of 232 chronic CD patients attending at Ambulatory of Chagas Disease of Hospital das Clínicas, Federal University of Paraná, were investigated [mean age 57 years; 130 (56%) females, 102 (44%) males, 176 (75.9%) Euro-, 44 (19%) Afro-Brazilian, 1 (0.4%) Asian, 11 (4.7%) Amerindian]. CD diagnosis was performed by two serological tests (ELISA and immunofluorescent antibody assay). Clinical findings were in accordance with those outlined by the Pan-American Health Organization (PAHO) and World Health Organization (WHO).1,2

Clinical details of the patients were obtained through medical records.

Table 3. Reconstructed CR1 haplotypes among CD patients and controls. NA: Not applicable. NS: Not significant.

| CR1 haplotypes (+4659/+4721/+4808/+4841/+4868/+4883) | Controls (n = 204) | CD Patient (n = 440) | Indeterminate (n = 174) | Cardiac (n = 154) | Digestive (n = 38) | Cardiac Digestive (n = 62) | With ECHO alteration (n = 48) | Without ECHO alteration (n = 148) | With Heart Failure (n = 102) | Heart Failure (n = 94) | Patient vs. Control p value; OR [95% CI] | Cardiac vs. Control p value; OR [95% CI] | Without ECHO alteration vs. Control p value; OR [95% CI] | Without Heart Failure vs. Control p value; OR [95% CI] |
|--------------------------------------------------------|---------------------|----------------------|------------------------|------------------|-------------------|--------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------|------------------------------------------|------------------------------------------|-------------------------------------------------|-----------------------------|
| CR1*AGAATA                                             | 3 (1.5)             | 23 (5.2)             | 7 (4)                  | 5 (3.2)          | 3 (7.9)           | 4 (6.4)                  | 6 (12.5)                    | 7 (4.7)                     | 13 (12.7)                   | 0               | p = 0.035; 3.99 [1.10–14.15]             | NS                         | NS                           | p = 0.03; 5.51 [1.17–25.8]                  |
| CR1*AGAGTG                                             | 2 (1)               | 8 (1.8)              | 3 (1.7)                | 1 (1.9)          | 2 (5.3)           | 0                        | 0                           | 3 (2)                       | 0                           | 3 (3.2)         | NS                         | NS                         | NS                           | p = 0.037; 11.14 [1.15–107]                 |
| CR1*AGAAAA                                             | 0                   | 2 (0.5)              | 0                      | 2 (1.3)          | 0                 | 0                        | 1 (0.7)                     | 1 (1)                       | 0                           | NA             | NA                         | NA                         | NA                           | NA                               |
| CR1*AAGAGG                                             | 0                   | 1 (0.2)              | 0                      | 0                | 1 (1.6)           | 0                        | 1 (0.7)                     | 0                           | 1 (1)                       | NA             | NA                         | NA                         | NA                           | NA                               |
| CR1*GGGAAA                                             | 0                   | 1 (0.2)              | 0                      | 0                | 1 (1.6)           | 0                        | 1 (0.7)                     | 0                           | 1 (1)                       | NA             | NA                         | NA                         | NA                           | NA                               |
| CR1*AAGATG                                             | 0                   | 1 (0.2)              | 1 (0.6)                | 0                | 0                 | 0                        | 0                           | 0                           | 0                           | NA             | NA                         | NA                         | NA                           | NA                               |
| CR1*AGATGG                                             | 0                   | 1 (0.2)              | 1 (0.6)                | 0                | 0                 | 0                        | 0                           | 0                           | 0                           | NA             | NA                         | NA                         | NA                           | NA                               |
| CR1*AGGATA                                             | 1 (0.5)             | 0                   | 0                      | 0                | 0                 | 0                        | 0                           | 0                           | 0                           | NA             | NA                         | NA                         | NA                           | NA                               |
and interviews. Patients younger than 18 years old with recent infection or suspected non-chagasic cardiomyopathy were excluded. Demographic and clinical characteristics of the distinct CD forms are shown in Table 4. Patients with cardiomyopathy were graded according to the cardiac insufficiency classification of the American Heart Association, adapted for CD66:

- A, altered electrocardiogram (ECG) and normal echocardiogram (ECHO), absence of cardiac insufficiency (CI);
- B1, altered ECG, LVEF > 45%, absence of CI;
- B2, altered ECHO, LVEF < 45%, absence of CI;
- C, altered ECG and ECHO, compensable CI;
- D, altered ECG and ECHO, refractory CI.

A group of 104 healthy Brazilians [mean age 51 years; 50 (48.1%) females, 54 (51.9%) males, 91 (87.5%) Euro-, 10 (9.6%) Afro-Brazilian, 2 (1.9%) Asian, 1 (1%) Amerindian] was used as control. All individuals from the control group were selected consecutively from a blood bank in the same geographic region as patients. Following Brazilian health regulations, the blood donors were screened for CD, syphilis, hepatitis B, hepatitis C, HIV and human T-cell lymphotropic viruses 1 and 2 using high sensitivity assays. Additionally, information about autoimmune diseases and cancer background was obtained during the pre-selection interview66. The study protocol was approved by the Ethics Committee of the Hospital de Clínicas, Federal University of Paraná (CEP/HC-UFPR n. 360.918/2013-08), and performed in accordance with relevant guidelines/regulations. Written informed consent was obtained from all patients and controls.

CR1 genotyping. In order to assess the distribution of the six functional CR1 exon 29 variants rs17259045 (g.207609362 A > G, p.N1540S), rs41274768 (g.207609424 G > A, p.V1561M), rs17047661 (g.207609511 A > G, p.K1590E), rs17047661 (g.207609544 A > G, p.R1601G), rs4844609 (g.207609571 T > A, p.T1610S) and rs6691117 (g.207609586 A > G, p.I1615V), the entire CR1 exon 29 including its intron-exon boundaries was directly sequenced only in 220 patients with chronic CD and 102 healthy control individuals. DNA from 12 patients and from two controls was degraded; therefore these individuals were excluded from further genetic analyses. Genomic DNA was extracted from buffy-coats using the QIAamp Blood mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer’s instructions. The CR1 reference sequence was retrieved from the Ensembl database (www.ensembl.org); primers targeting exon 29 of CR1 gene were designed manually, tested using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized commercially (Eurofins Genomics, Ebersberg, Germany). A fragment of 884 bp was amplified by polymerase chain reaction (PCR) using the CR1 locus specific primer pair CR1F (5′-TCT TCA TAA ATA ATG CCA GAA GTG G-3′) and CR1R (5′-TGC CAA TTT CAT AGT CCT TAT ACA C-3′). PCR amplifications were carried out in a 25 µl volume of reaction mixture containing 10 × PCR buffer, 3.0 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer, 1 unit of Taq polymerase (Qiagen) and 20 ng of genomic DNA on a TProfessional Basic Thermocycler (Biometra GmbH, Göttingen, Germany). Cycling parameters were initial denaturation at 94 °C for 5 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 55.5 °C for 30 seconds and elongation at 72 °C for 1 minute, and a final elongation step at 72 °C for 10 minutes. PCR fragments were stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, USA) and visualised in a 1.5% agarose gel. PCR products were purified using Exo-SAP-IT (USB-Affymetrix, Santa Clara, USA) and the purified products were directly used as templates for sequencing using the BigDye terminator cycle sequencing kit (v.3.1; Applied Biosystems, Texas, USA) on an ABI 3130XL DNA Analyzer. DNA polymorphisms were identified by assembling the sequences with the reference sequence of the CR1 (NM_000573) using the Geneious v9.1.4 software (Biomatters Ltd, Auckland, New Zealand) and reconfirmed visually from their respective electropherograms.

Quantification of soluble CR1 plasma levels. Measurements of erythrocyte CR1 plasma levels were performed in 221 patients and 102 controls using a commercial high-sensitivity ELISA kit (Human Complement Receptor 1/SEB123Hu, Cloud-Clone Corporation, Texas, USA) in accordance with the manufacturer’s instructions. The limit of detection was 0.312 ng/mL.

| Table 4. Baseline clinical parameters of the investigated study cohort. | Indeterminate (n = 92) | Cardiac (n = 87) | Digestive (n = 21) | Cardiodigestive (n = 32) | Controls (n = 104) |
|---|---|---|---|---|---|
| LVEF (%), [Range] | 57 [34–76] | 51 [34–90] | 57 [36–81] | 57 [37–73] | 51 [37–72] |
| Sex (Male/Female) | 34/58 | 46/41 | 15/16 | 18/14 | 54/50 |
| Ethnicity (Euro-Brazilian/Others) | 80/12 | 58/29 | 15/6 | 23/9 | 91/13 |
| Cardiac impairment (A,B,C,D) | NA | (27,22,36,02) | NA | (11,07,12,02) | NA |
| Erythrocytes (RBCs) (Million cells/L), [Range] | 4.7 [4.2–6.5] | 5.0 [3.6–6.0] | 4.8 [4.2–6.5] | 5.0 [4.3–5.6] | NA |
| Hemoglobin (mg/dL), [Range] | 14.4 [10.9–14.7] | 14.8 [9.0–17.7] | 14.6 [13.2–17.3] | 14.9 [12.9–17.4] | NA |
| uCRP levels (mg/dL), [Range] | 0.33 [0.08–3.77] | 0.34 [0.08–4.25] | 0.19 [0.09–0.38] | 0.34 [0.08–0.76] | NA |
| CR1 levels (ng/mL), [Range] | 11.75 [6.16–51.61] | 10.72 [6.18–37.93] | 9.46 [6.74–53.17] | 9.64 [7.34–40.97] | 17.25 [6.69–67.35] |
| LVEF (%), [Range] | 70 [35–84] | 65 [47–82] | NA | 66 [47–77] | NA |

SCIENTIFIC REPORTS | (2018) 8:526 | DOI:10.1038/s41598-017-18937-z
**Statistical Analysis.** CR1 plasma levels were compared between groups using nonparametric Kruskal-Wallis and Mann-Whitney tests. The distribution of each variable was assessed by the Shapiro-Wilk test. Multiple logistic regression was executed with adjustment for age, sex, and ethnic group. Multiple comparisons were corrected using a Benjamini-Hochberg procedure applying a false discovery rate of 0.10 and raw p-values that remained significant after this correction were considered in the study. Odds ratios (OR) and their 95% confidence intervals (CI) were calculated using the STATA software (v. 12.0, StataCorp, College Station, Texas, USA). Correlation analyses were performed by non-parametric Spearman’s rank coefficient tests. Allele frequencies were obtained by direct counting. Genotype and haplotype frequencies were analyzed by gene counting and expectation-maximum (EM) algorithms and the significance of deviation from Hardy-Weinberg equilibrium was tested using the random-permutation procedure as implemented in the Arlequin v. 3.5.2.2 software (http://lnb.unige.ch/arlequin). Linkage disequilibrium (LD) analysis was performed using Haploview v. 3.2 (http://broadinstitute.org/haploview). Possible associations of CR1 alleles, genotypes, and haplotypes with different clinical forms were evaluated with two-tailed Fisher exact tests. P-values < 0.05 were considered significant.

**References**

1. WHO. Chagas disease in Latin America: an epidemiological update based on 2010 estimates. Wkly. Epidemiol. Rec. Relev. épidémiologique Hebd. 6, 33–44 (2015).
2. Coura, J. J. et al. Control of Chagas disease. In World Health Organization - Technical Report Series 905, 1–99 (2002).
3. Schmunis, G. A. & Yadon, Z. E. Chagas disease: A Latin American health problem becoming a world health problem. Acta Trop. 115, 14–21 (2010).
4. Lee, B. Y., Bacon, K. M., Bottazzi, M. E. & Hotez, P. J. Global economic burden of Chagas disease: A computational simulation model. Lancet Infect. Dis. 13, 342–348 (2013).
5. WHO. Research priorities for Chagas disease, human African trypanosomiasis and leishmaniasis. World Health Organ. Tech. Rep. Ser. v–xi, 1–100 978 92 4 120975 5 (2012).
6. Cunha-Neto, E. & Chevillard, C. Chagas disease cardiomyopathy: Immunopathology and genetics. Mediators of Inflammation 2014, 683230 (2014).
7. Marin-Neto, J. A., Cunha-Neto, E., Maciel, B. C. & Simões, M. V. Pathogenesis of chronic Chagas heart disease. Circulation 115, 1109–1123 (2007).
8. Gomes, J. A. S. et al. Evidence that development of severe cardiomyopathy in human Chagas’ disease is due to a Th1-specific immune response. Infect. Immun. 71, 1185–93 (2003).
9. Geiger, A. et al. Escaping Deleterious Immune Response in Their Hosts: Lessons from Trypanosomatids. Front. Immunol. 7, 212 (2016).
10. Lidani, K. C. F., de Messias-Reason, I. J., Buvia, L. & Ambrosio, A. R. The Complement System: A Prey of Trypanosoma cruzi. Front. Microbiol. 8, 607 (2017).
11. Cestari, I., Evans-Osses, I., Schlabach, L. J., de Messias-Reason, I. & Ramirez, M. I. Mechanisms of complement lectin pathway activation and resistance by trypanosomatid parasites. Molecular Immunology 53, 328–334 (2013).
12. Romano, P. S. et al. Molecular and cellular mechanisms involved in the Trypanosoma cruzi/host cell interplay. JUBMB Life 64, 877–896 (2012).
13. Campo, V., Martins-Texeira, M. & Carvalho, I. Trypanosoma cruzi Invasion into Host Cells: A Complex Molecular Targets Interplay. Mini-Reviews Med. Chem. 16, 1084–1097 (2016).
14. De Souza, W., De Carvalho, T. M. U. & Barrias, E. S. Review on Trypanosoma cruzi: Host cell interaction. International Journal of Cell Biology 2010 (2010).
15. Cestari, I. et al. Role of early lectin pathway activation in the complement-mediated killing of Trypanosoma cruzi. Mol. Immunol. 47, 426–437 (2009).
16. Evans-Osses, I. et al. Differential ability to resist to complement lysis and invas host med cells mediated by MBL in R4 and 860 strains of Trypanosoma cruzi. FERB Lett. 588, 956–961 (2014).
17. Holers, V. M. Complement and its receptors: new insights into human disease. Annu. Rev. Immunol. 32, 433–459 (2014).
18. Luz, P. R., Miyazaki, M. I., Neto, N. C., Nisihara, R. M. & Messias-Reason, I. High levels of mannose-binding lectin are associated with the risk of severe cardiomyopathy in chronic Chagas Disease. Cardiology 143, 448–450 (2010).
19. Lidani, K. C. F. et al. Is pentraxin 3 a cardiovascular marker in patients with chronic Chagas Disease? International Journal of Cardiology 190, 233–235 (2015).
20. Liu, D. & Niu, Z.-X. The structure, genetic polymorphisms, expression and biological functions of complement receptor type 1 (CR1/CD35). Immunopharmacol. Immunotoxicol. 31, 324–35 (2009).
21. Ghiran, I. et al. Complement Receptor 1/CD35 Is a Receptor for Mannan-Binding Lectin. J. Exp. Med. 192, 1797–1808 (2000).
22. Jacquet, M. et al. Deciphering Complement Receptor Type 1 Interactions with Recognition Proteins of the Lectin Complement Pathway. J. Immunol. 190, 3721–3731 (2013).
23. Krych-Goldberg, M. & Atkinson, J. P. Geographical distribution of complement receptor type 1 variants and their associated disease risk. PLoS One 12, e0175973 (2017).
24. Rosenthal, L., Sutterwala, F., Kehrli, M. & Mosser, D. Leishmania major-human macrophage interactions: cooperation between Mac-1 (CD11b/CD18) and complement receptor type 1 (CD35) in promastigote adhesion. Infect. Immun. 64, 2206–2215 (1996).
25. Dominguez, M., Moreno, I., Lopez-Trascasa, M. & Torrado, A. Complement Interaction with Trypanosomatid Promastigotes in Normal Human Serum. J. Exp. Med. 195, 451–459 (2002).
26. Odera, M., Otieno, W., Adhiamo, C. & Stoute, J. A. Dual role of erythrocyte complement receptor type 1 in immune complex-mediated macrophage stimulation: Implications for the pathogenesis of Plasmadium falciparum malaria. Clin. Exp. Med. 166, 201–207 (2011).
27. Carroll, M. V., Lack, N., Sim, E., Kraput, A. & Sim, R. B. Multiple routes of complement activation by Mycobacterium bovis BCG. Mol. Immunol. 46, 3367–3378 (2009).
28. Fitness, J., Tosh, K. & Hill, A. V. S. Genetics of susceptibility to leprosy. Genes Immun. 3, 441–453 (2002).
29. Beck, Z. et al. Human erythrocytes selectively bind and enrich infectious HIV-1 virions. PLoS One 4, e8297 (2009).
30. Horakova, E. et al. Complement mediates the binding of HIV to erythrocytes. J. Immunol. 173, 4236–4241 (2004).
31. Wang, P. S. et al. Acquired but reversible loss of erythrocyte complement receptor 1 (Cr1, Cd35) and its longitudinal alteration in patients with severe acute respiratory syndrome. Clin. Exp. Immunol. 139, 112–9 (2005).
32. Seregin, S. S. et al. CR1/2 is an important suppressor of Adenovirus-induced innate immune responses and is required for induction of neutralizing antibodies. Gene Ther. 16, 1245–1259 (2009).
33. Mehlhop, E. et al. Complement activation is required for induction of a protective antibody response against West Nile virus infection. J. Virol. 79, 7466–77 (2005).
36. Senbagavalli, P. et al. Reduced erythrocyte CR1 levels in patients with pulmonary tuberculosis is an acquired phenomenon. *Clin. Immunol. 128*, 109–115 (2008).

37. Tausk, F., Hoffmann, T., Schreiber, R. & Gigli, I. Leprosy: altered complement receptors in disseminated disease. *J. Invest. Dermatol. 85*, 58–61 (1985).

38. Di Bona, D. et al. Soluble complement receptor type 1 (sCR1) in chronic liver diseases: Serum levels at different stages of liver diseases. *Clin. Exp. Immunol. 114*, 102–105 (1998).

39. Khera, R. & Das, N. Complement Receptor 1: disease associations and therapeutic implications. *Mol. Immunol. 46*, 761–72 (2009).

40. Sivasankar, B. et al. Levels of plasma soluble complement receptor 1 (sCR1) in normal Indian adult population. *Indian J. Clin. Biochem. 14*, 237–40 (1999).

41. Boiocchi, C. et al. CR1 genotype and haplotype involvement in coronary artery disease: The pivotal role of hypertension and dyslipidemia. *Int. J. Mol. Med. 24*, 181–187 (2009).

42. Luz, P. P. et al. Genetically Determined MBL Deficiency Is Associated with Protection against Chronic Cardiomyopathy in Chagas Disease. *PLoS Negl. Trop. Dis. 10*, e0004257 (2016).

43. Luz, P. R. et al. Association of L-Ficolin Levels and FCN2 Genotypes with Chronic Chagas Disease. *PLoS One 8*, e60237 (2013).

44. Boldt, A. B. W., Luz, P. R. & Messias-Reason, I. J. T. MASP2 haplotypes are associated with high risk of cardiomyopathy in chronic Chagas disease. *Clin. Immunol. 140*, 63–70 (2011).

45. Mora, G. Chagas cardiomyopathy. *E-Journal Cardiol. Pract. - Eur. Soc. Cardiol. 14* (2016).

46. Bjerre, M., Hansen, T. & Flyvbjerg, A. Complement Activation and Cardiovascular Disease. *Horm. Metab. Res. 40*, 626–634 (2008).

47. Carter, A. M. Complement activation: an emerging player in the pathogenesis of cardiovascular disease. *Scientifica (Cairo). 2012*, 402783 (2012).

48. Speidl, W. S. et al. Complement component C5a predicts future cardiovascular events in patients with advanced atherosclerosis. *Eur. Heart J. 26*, 2294–2299 (2005).

49. Oksjoki, R. et al. Association between complement factor H and proteoglycans in early human coronary atherosclerotic lesions: Implications for local regulation of complement activation. *Arterioscler. Thromb. Vasc. Biol. 23*, 630–636 (2003).

50. Picecchi-Martí, M. et al. Immunoablation by complement C9: a tool for early diagnosis of myocardial infarction and application in forensic medicine. *J. Forensic Sci. 46*, 328–34 (2001).

51. Carter, A. M., Prasad, U. K. & Grant, P. J. Complement C3 and C-reactive protein in male survivors of myocardial infarction. *Horm. Metab. Res. 46*, 2294–2299 (2005).

52. Kullo, I. J. *Cardiovasc. Res.* 19–62, https://doi.org/10.1201/9780849350368.ch2 (CRC Press, 2005).

53. Kullo, I. J. et al. *Complement C3 and C-reactive protein in myocardial infarction* (CRC Press, 2005).

54. Kullo, I. J. & Prasad, U. K. Complement activation: an emerging player in the pathogenesis of cardiovascular disease. *Scientifica (Cairo). 2012*, 402783 (2012).

55. Moulds, J. M. et al. Complement Activation and Cardiovascular Disease. *Horm. Metab. Res. 40*, 626–634 (2008).

56. Carter, A. M. Complement activation: an emerging player in the pathogenesis of cardiovascular disease. *Scientifica (Cairo). 2012*, 402783 (2012).

57. McElroy, J. J. *Trans. Med.* 19–62, https://doi.org/10.1201/9780849350368.ch2 (CRC Press, 2005).

58. McElroy, J. J. et al. Immunoablation by complement C9: a tool for early diagnosis of myocardial infarction and application in forensic medicine. *J. Forensic Sci. 46*, 328–34 (2001).

59. Carter, A. M., Prasad, U. K. & Grant, P. J. Complement C3 and C-reactive protein in male survivors of myocardial infarction. *Atherosclerosis 203*, 538–543 (2009).

60. Pedersen, E. D., Waje-Andreassen, U., Vedeler, C. A., Aamoedt, G. & Møllnes, T. E. Systemic complement activation following human acute ischaemic stroke. *Clin. Exp. Immunol. 137*, 117–122 (2004).

61. Noumsi, G. T. et al. Levels of plasma soluble complement receptor type 1 in patients receiving thrombolytic therapy for acute myocardial infarction. *J. Am. Heart J.* 132, 268–277 (1996).

62. Jiao, B. et al. Soluble complement receptor type 1 (sCR1) in chronic liver diseases: Serum levels at different stages of liver diseases. *Clin. Exp. Immunol. 114*, 102–105 (1998).

63. Khera, R. & Das, N. Complement Receptor 1: disease associations and therapeutic implications. *Mol. Immunol. 46*, 761–72 (2009).

64. Yu, X. et al. Tag SNPs in complement receptor-1 contribute to the susceptibility to non-small cell lung cancer. *Clin. Exp. Immunol. 114*, 102–105 (1998).

65. PAHO. El Salvador - Ministério de la Salud Publica y Asistencia Social: Norma Técnica de Prevención y Control de la Enfermedad de Chagas: II Consenso Brasileiro em Doença de Chagas, 2015.
