The Clinical Value of Ficolin-3 Gene Polymorphism in Rheumatic Heart Disease. An Egyptian Adolescents Study

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Abstract

Background: The innate immunity molecules against *Streptococcus pyogenes* infections include ficolin-3 that was thought to have a role in autoimmune diseases resulting from this infection such as rheumatic fever (RF) which goes on and leads to its most serious sequel, rheumatic heart disease (RHD). We aimed in this study to disclose if there is an association between ficolin-3 gene polymorphisms (rs4494157 and rs10794501) and RF with or without RHD for the first time in Egyptian adolescents.

Methods: This study was carried out on 160 RF patients; eighty patients had RHD and eighty didn't have RHD. Besides, eighty ethnically and age-matched healthy subjects were selected as control. Ficolin-3 gene polymorphisms (rs4494157 and rs10794501) were genotyped by TaqMan® allelic discrimination assay. Serum ficolin-3 was quantitatively determined by ELISA.

Results: Regarding ficolin-3 gene polymorphisms; no differences in the frequency of rs10794501 were found between the investigated groups, while polymorphism of rs4494157 showed a significant correlation between AA homozygous genotype, high serum ficolin-3, and RHD risk.

Conclusion: Elevated serum ficolin-3 and carriage of AA genotype of rs4494157 appeared to be involved in RF and RHD pathogenesis and may be predictive tools for early recognition of RHD prone RF patients, and subsequent early prophylactic interventions.

Background

Rheumatic fever (RF) is a public health care burden in low-income and developing countries, where the highest prevalence of RF exists, especially in children and young adults. RF is a consequence of recurrent group-A *Streptococcus pyogenes* (GAS) pharyngitis as an immune-mediated complication in individuals genetically susceptible [1, 2].

The major manifestations that indicate the involvement of target tissue in RF include rheumatic arthritis, rheumatic carditis, Sydenham's chorea, and subcutaneous nodules. Repeated or severe episodes of RF lead to permanent harm to the heart valves, bringing about rheumatic heart disease (RHD); the well-known acquired cardiovascular disease in youth. In 2015, the prevalence of RHD was estimated to be 33,194,900 patients in RHD- endemic countries, while there were only 221,600 patients in non- endemic countries [3]. The evaluated RHD prevalence in nations with low-income and middle-income ranges from 2.7 to 51 cases per 1,000 population for clinically apparent and clinically silent RHD sequentially [3]. A high prevalence (31 per 1000 children) of RHD in schoolchildren in Egypt and other African countries was reported [4].

RF and RHD are multifactorial disorders that involve multiple environmental and genetic factors, but not yet fully elucidated [5]. The pathogenesis of RF and RHD is strongly dependent on autoimmunity. Autoantibodies produced from molecular mimicry between proteins of heart tissue and GAS mediate tissue damage. It was found that GAS molecules (as N-acetyl-β-D-glucosamine GlcNAc and M protein)

display across-reactivity with valve and myocellular contractile proteins of the host. Ficolins represent important pattern-recognition molecules of the complement system that can identify GlcNAc; one of the most immunodominant antigens related to the cell wall of GAS [2, 6–8].

A double-edged action had been suggested to the complement system in both GAS infection defense and in autoimmunity development in RHD. This system immediately ripostes to any pathogen and plays an important role in building a bridge between responses of both adaptive and innate immunity [9–13].

Lectin pathway is the main complement pathway involved in immunity against capsular polysaccharide antigen of *Streptococcus pyogenes*, which can be activated by ficolins and collectins. Mannose-binding lectin (MBL) is one of the important molecules of collectins. The oligomeric structures of ficolins exist in a basic homotrimer, in which each chain is built from a collagenous strand and a fibrinogen-like recognition domain. Concerning ficolin oligomers, they are bounded to MBL associated serine proteases (MASP-1 and MASP-2). Moreover, ficolin binds certain patterns of acetylated residues on the surface of pathogens or altered cells [7, 14, 15]. MASP-1, at this point auto-activates and trans-activates MASP-2 leading to cleavage of downstream complement constituents [15, 16].

The lectin pathway, along with both classical and alternative pathways intersects at the cleavage of C3 and C5 followed by the opsonization of C3b and pathogen phagocytosis or eradication of pathogen by the arrangement of a membrane attack complex (MAC) [17].

Up till now, three types of human ficolin have been recognized: the ficolin-1 (M ficolin), the ficolin-2 (L ficolin), and finally the ficolin-3 (H ficolin, Hakata antigen) [8]. Ficolin-3 is synthesized in both the liver and lungs, representing the most abundant circulating ficolins [18]. Serum ficolin-3 has been reported to be widely variable among healthy individuals, which may be attributed to the genetic makeup [19–21]. Functionally, like other ficolins, the ficolin-3 has been shown to interact with the MASPs enabling the activation of the complement system [22, 23]. Moreover, ficolin-3 has been found to have the highest complement system activating potential over the other lectin pathway initiating molecules [24].

Low serum ficolin-3 level has been recognized to be associated with the pathogenesis of sarcoidosis [25], infections associated with chemotherapy [26], and chronic heart failure [27]. While high levels of serum ficolin-3 were found to be associated with ovarian cancer [28] and systemic lupus erythematosus [29] and seem to be a risk factor for shorter graft survival in kidney transplantation post-operation [30].

Ficolin-3 is encoded by the *FCN3* gene which is located on chromosome 1p36 and contains eight exons. Few studies showed an association between collectin and ficolin genes polymorphisms and infectious and autoimmune diseases [31–36]. It was shown that there are some associations between gene polymorphisms of MBL [37–40], Ficolin-1 [16], Ficolin-2 [41] [42], and MASP-2 [43] with RF. However, the impact of the ficolin-3/*FCN3* gene on RF and RHD is currently obscure.

Despite being a preventable disease, RHD may proceed silently until patients are presented as debilitating HF cases. In this case, surgery is the only possible choice for treatment [44], and deadly outcomes

ultimately occur [3]. RHD is a disease of poverty. So, patients living in poor nations have restricted or no access to costly heart medical procedures [45]. Currently, there are no markers for RF or RHD prediction. To avoid this dangerous sequel, it is very critical to have a predictive tool that could help in finding out the most vulnerable patients to RHD to get their medical follow-up.

This study aimed to investigate for the first time the association of *FCN3* gene polymorphisms (rs4494157 and rs10794501) as well as ficolin-3 serum levels with the susceptibility of RF and RHD development in Egyptian adolescents.

Subjects And Methods

Patients and Control Subjects

This study was performed on 240 subjects that were grouped as follows. The first group consisted of 80 RF patients without RHD. The second group included 80 RF patients with RHD. While in the third group, eighty apparently healthy volunteers matching with the patients for age, sex, ethnic and geographic origin were selected as controls. RF patients were recruited from the Cardiology Outpatient Clinic, Children Hospital, Cairo University, Egypt. A free informed consent form was signed by parents of both controls and patients. Approval of this study by the ethical committee of Children Hospital, School of Medicine, Cairo University, Egypt has been obtained. Patients with any infections, acute RF, infective endocarditis, or any other inflammatory disorders were excluded from this study. All enrolled patients had a clinical history of RF. The presence of mitral valve regurge in patients with RHD was confirmed by an echocardiogram.

Methods

A venous blood sample was withdrawn from all enlisted subjects and then separated into two tubes. The first tube was allowed to clot, then the obtained serum was collected after centrifugation then divided into aliquots and kept at -80 C° until utilized in the concentration determination of ficolin-3 by enzyme-linked immunosorbent assay (ELISA) utilizing commercial kits (Ray Bio Kit Inc., Georgia, USA) based on manufacturer's instructions and recommendations.

Extraction of Genomic DNA (gDNA) from the whole blood sample of the 2nd tube was performed depending on Gene JET™ Whole Blood DNA Purification Mini Kit (Thermo Fisher Scientific Inc., USA). NANODROP™ 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA) was utilized to determine the gDNA purity and concentration (A260/280) for each sample. Polymorphisms at (rs4494157 and rs10794501) in the *FCN3* gene were typed by real-time polymerase chain reaction (RT-PCR) utilizing TaqMan® allele discrimination assay (Applied Biosystems, CA, USA). The designation of primers and probes was carried out by Applied Biosystems (ID C__11597746_10) and (IDC__31148020_10) respectively.

Statistical Analysis

GraphPad Prism 6.2 (GraphPad Software, San Diego, USA) was utilized to perform the statistical analysis of our data. Normality distribution of variables was checked using D'Agostino-Pearson Omnibus test, where normally distributed variables were presented as mean ± SE while we used median (inter-quartile range) to represent the skewed distributed variables. Kruskal-Wallis test was used to compare between all groups followed by Dunn's test. Genotypes distribution for the polymorphism was checked for deviation from the Hardy-Weinberg equilibrium (HWE) and any deviations between observed and expected frequencies were examined for detection of significance depending on the χ^2 test. Besides, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Moreover, serum ficolin-3 best cutoff value for RHD was defined using the ROC curve analysis. A p-value < 0.05 was considered statistically significant.

Results

The demographic data and serum concentration of ficolin-3 in individuals enrolled in the study were presented in Table 1. Serum ficolin-3 levels were significantly increased in RF patients with and without RHD Vs controls, while there was no significant difference between RF with and without RHD. The diagnostic power of serum ficolin-3 to differentiate RHD patients from control subjects and RF patients without RHD collectively was revealed depending on the analysis of the ROC curve. The critical ficolin-3 serum level associated with RHD risk was 17685 ng/ml (AUC = 0.783, SE = 0.028, 95% CI = 0.727 to 0.838, p < 0.0001, 68.8% sensitivity and 68.1% specificity) as illustrated in Fig. 1.

Table 1
Demographic data and serum levels of ficolin-3 in all studied groups

Characteristics		Controls (n. = 80)	RF (n. = 160)	
			Without RHD (n. = 80)	With RHD (n. = 80)
Gender	Female n. (%)	48 (60.0)	46 (57.5)	48 (60.0)
	Male n. (%)	32 (40.0)	34 (42.5)	32 (40.0)
Age (years)		15.2 ± 0.29	14.5 ± 0.43	14.3 ± 0.33
Ficolin-3 (ng/ml)		8370 ± 100.2	18258 ± 136.5 [a]	18665 (17535–19640) [a]
Data are presented as n. (%), mean ± SE or median (inter-quartile range).				
a: significant from control group at *P* < 0.05.				

The genotypic and allelic results of *FCN3* single nucleotide polymorphism (SNP) (rs4494157) analysis for the studied groups were listed in Table 2. The rs4494157 genotype distribution showed no deviation from Hardy–Weinberg equilibrium (p > 0.05). The genotypic distribution revealed a higher frequency of the heterozygous CA only and CA/AA genotypes in RF patients with and without RHD when compared to controls. Also, the homozygous variant AA genotype showed higher frequency in RF with RHD as

compared to controls. Besides, a higher frequency of the A allele was observed in RF patients with and without RHD when compared to the controls.

Table 2
Distribution of *FCN3* genotypes (rs4494157) in controls and patients with RF

	Control n (%)	RF without RHD n (%)	RF with RHD n (%)	OR (95% CI)	*P*
Genotype distribution					
CC	64 (80)	49 (61.25)	43 (53.75)	1.00	-
CA	10 (12.5)	20 (25)	23 (28.75)	2.61 (1.12–6.08) [a]	0.026 [a*]
				3.42 (1.48–7.91) [b]	0.005 [b*]
				1.31 (0.63–2.71) [c]	0.580 [c]
AA	6 (7.5)	11 (13.75)	14 (17.5)	2.39 (0.83–6.93) [a]	0.121 [a]
				3.47 (1.24–9.74) [b]	0.026 [b *]
				1.50 (0.60–3.50) [c]	0.500 [c]
CA/AA	16 (20)	31 (38.75)	37 (46.25)	2.53 (1.24–5.14) [a]	0.016 [a *]
				3.44 (1.70–6.95) [b]	0.0007 [b *]
				1.36 (0.72–2.55) [c]	0.424 [c]
Allele frequencies					
C	138 (86.25)	118 (73.75)	109 (68.12)	1.00	-
A	22 (13.75)	42 (26.25)	51 (31.88)	2.23 (1.26–3.95) [a]	0.008 [a *]
				2.93 (1.68–5.14) [b]	0.0002 [b *]
				1.31 (0.81–2.13) [c]	0.325 [c]

OR: odds ratio; CI: confidence intervals; a: RF patients without RHD *vs*. control; b: RF patients with RHD *vs*. control; c: RF patients with RHD *vs* RF patients without RHD; *: Statistically significant different at $P < 0.05$ using Fisher exact test.

The genotype distribution for *FCN3* SNP (rs10794501) showed no deviation from HWE. All patients did not show any significant differences in the frequency of all genotypes and alleles when compared to controls and when compared to each other, as illustrated by Table 3.

Table 3
Distribution of *FCN3* genotypes (rs10794501) in controls and patients with RF

	Control n (%)	RF without RHD n (%)	RF with RHD n (%)	OR (95% CI)	*P*
Genotype distribution					
TT	58 (72.5)	51 (63.75)	54 (67.5)	1.00	-
TA	17 (21.25)	21 (26.25)	16 (20)	1.40 (0.67–2.95) [a]	0.452 [a]
				1.01 (0.46–2.20) [b]	1.00 [b]
				0.72 (0.34–1.53) [c]	0.447 [c]
AA	5 (6.25)	8 (10)	10 (12.5)	1.82 (0.56–5.92) [a]	0.385 [a]
				2.15 (0.69–6.69) [b]	0.271 [b]
				1.18 (0.43–3.23) [c]	0.803 [c]
TA/AA	22 (27.5)	29 (36.25)	26 (32.5)	1.50 (0.77–2.93) [a]	0.309 [a]
				1.27 (0.64–2.50) [b]	0.605 [b]
				0.85 (0.44–1.63) [c]	0.739 [c]
Allele frequencies					
T	133 (83.1)	123 (76.9)	124 (77.5)	1.00	-
A	27 (16.9)	37 (23.1)	36 (22.5)	1.48 (0.85–2.58) [a]	0.208 [a]
				1.43 (0.82–2.49) [b]	0.261 [b]
				0.96 (0.57–1.63) [c]	1.00 [c]

OR: odds ratio; CI: confidence intervals; a: RF patients without RHD *vs*. control; b: RF patients with RHD *vs*. control; c: RF patients with RHD *vs* RF patients without RHD.

Table 4 showed the differences in serum ficolin-3 levels according to the *FCN3* (rs4494157) genotypes in all patients. Results revealed that patients carrying the A allele (CA + AA) were associated with significantly high serum ficolin-3 as compared to those carrying the CC genotype. However, as illustrated in Table 5, the classification of the RF group according to the *FCN3* (rs10794501) genotypes showed no significant differences between TT and TA + AA genotypes regarding serum ficolin-3 level. So, no association between ficolin-3 and *FCN3* gene polymorphism (rs10794501) was found.

Table 4
Age and ficolin-3 levels of RF patients according to *FCN3* (rs4494157) genotypes

	RF patients	
	CC	CA + AA
Age (years)	14.44 ± 0.30	14.35 ± 0.55
Ficolin-3 (ng/ml)	18059 ± 119.2	19215 (17928–19760)[a]
Data are presented in mean ± SE or median (inter-quartile range).		
a: significant difference between CA + AA and CC at $P < 0.05$.		

Table 5
Age and ficolin-3 levels of RF patients according to *FCN3* (rs10794501) genotypes

	RF patients	
	TT	TA + AA
Age (years)	14.44 ± 0.30	14.35 ± 0.55
Ficolin-3 (ng/ml)	18630 (17495–19680)	18203 ± 159.7
Data are presented in mean ± SE or median (inter-quartile range).		

Discussion

The autoimmune reaction due to the molecular similitude between antigens of *Streptococcus pyogenes* and proteins in the tissues of joints, heart, and central nervous system is the primary speculation underlying the development of RF and RHD [2]. Few genetic polymorphisms in the particles that participate in the immune responses have been contributed in RF and RHD vulnerability [46]. The activation cascade of the complement proteins is the first-line defense barrier against *Streptococcus pyogenes* infections. As of their significant role in disposing of rheumatic etiological agents, the deficiency of lectin pathway molecules is considered as responsible for the seriousness and vulnerability to rheumatic morbidities [47, 48]. The current writing about the role of MBL and ficolins in the pathogenesis of RF and RHD is as yet rare; however, their double function regarding RF has been hypothesized [11].

Pathogens are recognized and bound to ficolins, resulting in their neutralization and opsonization, followed by activation of the complement lectin pathway [49]. Although ficolins together with MBL are fundamentally similar both in terms of structure and function, these proteins likely have diverse roles in immunity. Ficolin-3 can actuate the lectin pathway of complement to a much higher degree than ficolin-1,

ficolin-2, and MBL. Exclusively among ficolins, ficolin-3 was highly resistant to collagenase-treatment as compared with the other initiators of the lectin complement pathway [50].

Unfortunately, studies on the significance of ficolins in RF and RHD are insufficient, despite their obvious contribution in activation of the innate immune response through complements and in autoimmunity [51].

Few previous studies on *FCN1* and *FCN2* genes have found that some polymorphisms of both genes could give a protective role against RF, by empowering bacterial elimination in addition to activation of the expression of these genes leading to an increase in the production of their proteins. Contrarily, they could also make the patients more susceptible to symptoms of RHD, probably due to contribution in tissue injury and chronic inflammation, in this way emphasizing the double action of ficolin-1 and ficolin-2 in both cases [16, 41, 42].

Up till now, the role of *FCN3* gene polymorphism in RF and RHD pathogenesis remains unknown. As far as we could possibly know, this is the first study to investigate *FCN3* gene polymorphisms (rs4494157 and rs10794501) together with their related genotypes and levels of serum ficolin-3 in patients suffering from RF and RHD.

In the current study, the significant high serum level of ficolin-3 in patients suffering from RF with and without RHD in comparison with control reflects its role in complements initiation and subsequent pathogenesis of RF and RHD. There have been no reports concerning the relationship between ficolin-3 and RF but several findings are indicating that high levels of ficolin-3 may contribute to the induction of inflammation as in diabetic retinopathy [52], SLE [29], leprosy [53], ovarian cancer [28], acute leukemia [26] and associated with post-operative graft loss in kidney transplantation [30].

Based on the increase in inflammation development associated with high levels of ficolin-3, It was assumed that the involvement of ficolin-3 in immune evasion of *Streptococcus pyogenes* in RF and RHD patients is as a result of its anti-opsonic response to complements overactivation [54].

Interestingly, lectin pathway activators, including MBL, both ficolin-1 and 2 were appeared to bind to *Streptococcus pyogenes* leading to MASPs activation [11]. Although no direct binding of ficolin-3 on *Streptococcus pyogenes* was found, it is known that the *Streptococcus pyogenes* cell walls contain long polymers of GlcNAc, that is a target for ficolins [55] and could, therefore, be a potential ligand for ficolin-3 also. So, our findings open a new window to study the potential interaction between ficolin-3 and *Streptococcus pyogenes*.

Given the (rs4494157), we observed that higher ficolin-3 levels were also associated with certain genotypes of *FCN3* that contain the A allele in intron 7. Interestingly, intron 7 contains CpG islands and enriched for typical modifications of histone that are known to characterize active enhancers [56, 57].

Several regulatory proteins (such as SPI1-Spleen focus forming virus (SFFV), CTCF-CCCTC-binding factor, EGR1-Early growth response protein 1 and Proviral Integration 1) bind to this intronic area, as shown by

chromatin immune-precipitation technique in several cell lines (such as HMC-cardiac myocytes, NHL-lung fibroblasts, and BJ-skin fibroblast). The variants within this sequence may increase the activity of enhancer in light to inflammation signals, resulting in stimulated gene transcription and higher levels of its protein [53].

Additionally, the possibility of regulatory proteins binding in this site could modify the alternative splicing of exon 4, whose inclusion in the most abundant *FCN3* transcript leads to a longer collagenous tail [53].

The most important result in this study was related to the *FCN3* A allele (rs4494157). Our finding suggests that this allele may be a risk factor for the progression of RF to its chronic form. Thus, patients conveying the *FCN3* A allele may be at high risk for recurrent infection, and a higher likelihood to develop in RHD. Consequently, early identification, carefully monitoring should be given for those patients. Besides, clinicians must confirm adherence of those patients to secondary prophylaxis intervention [58]. Actually, secondary prophylaxis adherence of RF patients is typically poor, especially in young people which was perceived as the principal explanation for RF repeats and RHD advancement [58, 59].

Significant differences in the distribution of MBL2 and *FCN3* alleles and genotypes have been identified among the population from various continents. These distinctive genetic patterns may almost certainly affect serum MBL and ficolin-3 levels, accordingly modifying worldwide susceptibility for the disease [60, 61]. What's more, the presence of the C allele in controls in highly significant patterns as compared to RF patients could show that the presence of the C allele may have a defensive action against the occurrence of RF and RHD. Moreover, these data propose that the cardiac manifestations development of RF is related to high ficolin-3 levels and its linked genotypes, also, that this relationship is a direct result of a certain mechanism related to *FCN3* gene polymorphism and not related to an acute-phase reaction.

In interpreting the results of our study, some restrictions should be addressed. First, SNPs selection was dependent on what we have found in the literature. However, there are many SNPs that could be highly prominent in ethnics included in this study. Second limitation, the sample size was not huge enough to clarify a more extensive picture for *FCN3* genotype distribution among Egyptian adolescents. So, to verify these findings, further larger sample-based studies are recommended in Egyptians and Mediterranean ethnics. Third, the present study does not include long-term patients' follow-up, so it is difficult to recognize which patients develop RHD and its associated risk factors.

Conclusion

Together with serum ficolin-3, RHD genetic susceptibility screening for *FCN3* AA genotype (rs4494157), may provide clinically useful genetic-based risk prediction marker to RHD liable RF patients, and consequent echocardiographic screening, and prophylactic intervention to prevent the disease burden, especially in resources constrained nations.

Abbreviations

Full term	Abbreviation
Complement	C
Confidence intervals	Cis
Cytosine - guanine dinucleotide	CpG
Enzyme linked immunosorbent assay	ELISA
Ficolin gene	*FCN*
Genomic DNA	gDNA
Group-A Streptococcus pyogenes	GAS
Hardy–Weinberg equilibrium	HWE
Heart failure	HF
Mannose-binding lectin	MBL
MBL associated serine proteases	MASP
Membrane attack complex	MAC
N-acetyl-β-D-glucosamine	GlcNAc
Odds ratios	ORs
Pattern-recognition receptor	PRR
Real-time polymerase chain reaction	RT-PCR
Rheumatic fever	RF
Rheumatic heart disease	RHD
Single nucleotide polymorphism	SNP
Systemic lupus erythematosus	SLE

Declarations

Ethics Approval and Consent to Participate:

This study has been approved by the ethical committee of Children Hospital, School of Medicine, Cairo University, Egypt.

Consent for Publication: Not Applicable.

Availability of Data and Material:

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing Interests:

The authors declare that they have no competing interests.

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Authors' Contributions:

HH and AF were responsible for patients' diagnosis, clinical recruitment and sampling. MG, AE, and EK carried out the practical part of the work. AAE and AA contributed in statistical analysis and data collection. MG, AE, and ME contributed equally in literature collection, manuscript writing, and thorough reviewing. All authors have read and approved the final manuscript.

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Figures

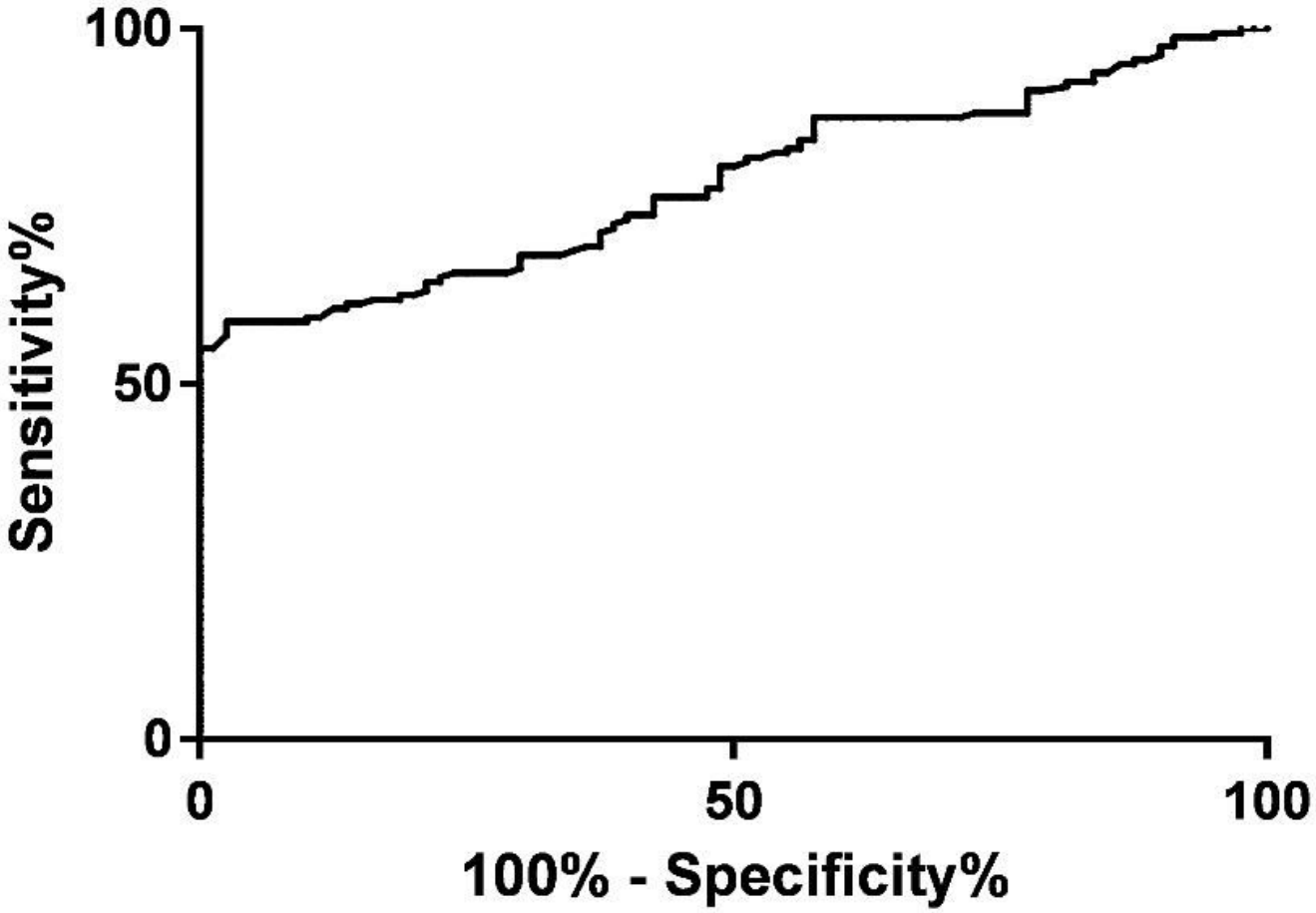

Figure 1

ROC curve of serum ficolin-3 in RF with RHD patients vs. controls and RF without RHD.