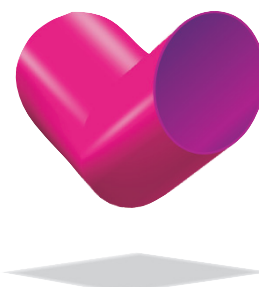


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Editorial

The 2012 JCBC issue represents the 15th year of its publication.

Many important developments have taken place during this time, in particular technical improvements like transcatheter valve implantation or advances in electrophysiology have benchmarked recent developments. Pharmacologically, the recent years have brought about only minor changes. Some improvements have been seen in anti-coagulant therapy, ivabradin as an If-current blocker seems to be the only new class of cardiovascular pharmacotherapeutics apart from the renin-antagonist aliskiren.

Prospectively, we expect major advances on the technical side, in particular, as far as new devices and improved non-invasive diagnostic techniques are concerned. New demands will be put on training and specialization: the expected developments will ask for new types of “cardiolo-

gists” such as interventional cardiac surgeons, cardiac team specialists, cardio-microbiologists, cardiac gene therapists etc.

While on the one hand vast fields like cardiology and cardiac surgery seem to amalgamate, cardiology itself becomes more and more fractioned and sub-specialties like rhythmology or interventional cardiology seem to grow into separate specialties.

We all look forward with great interest to the upcoming developments to be seen within the next 15 years of this journal.

Robert Gasser, Managing Editor



R. Gasser

Expansion of CD4⁺CD28^{null} T Lymphocytes Contributes to Coronary Artery Diseases in Rheumatoid Arthritis Patients

M. E. Ansary¹, E. A. Ghaffar², N. M. Gendy¹, N. S. Hasan², M. M. Sedkey³

Background: The expanded CD4⁺ clonotype T cell subset lacking surface CD28 has been suggested to predispose rheumatoid arthritis (RA) patients to develop more aggressive disease including extra-articular manifestations and CAD. **Patients and Methods:** The number of circulating CD4⁺CD28^{null} T cells was evaluated in 42 RA and 10 control subjects by 2-color FACSort flow cytometer, patients underwent evaluation of joint involvement, extra-articular manifestations, and CAD association. **Results:** The frequency of CD4⁺CD28^{null} cells was significantly higher in patients than in control subjects. RA patients with persistent expansion of circulating CD4⁺CD28^{null} cells had more marked increase of extra-articular manifestation ($P = 0.000$) and CAD ($P = 0.000$). Expansion of circulating CD4⁺CD28^{null} T cells correlated significantly with elevated ESR, triglycerides, and seronegative RF. **Conclusion:** Circulating CD4⁺CD28^{null} T lymphocytes are increased in RA. Patients with persistent CD4⁺CD28^{null} T cell expansion show more extra-articular manifestation and CAD. These findings suggest that increased CD4⁺CD28^{null} T cell expansion, ESR, and triglyceride levels together with seronegative RF can serve as markers to help identify patients with rheumatoid arthritis who are at risk of developing CAD. Moreover, the correlation between CD4⁺CD28^{null} T cell expansion, ESR, and triglyceride levels together with seronegative RF may be of interest for possible innovative predictive and diagnostic strategies in cardiovascular diseases. *J Clin Basic Cardiol* 2012; 15 (online): 2–6.

Key words: CD4⁺, CD28^{null}, RA, extra-articular manifestations, CAD

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by uncontrolled proliferation of synovial tissue and a wide array of multi-system affection [1].

Rheumatoid arthritis is one of the best characterized autoimmune diseases associated with increased frequencies of CD4⁺CD28^{null} T cells [2–4]. Studies in patients with RA showed CD4⁺CD28^{null} T cells to be increased in > 1/3 of patients [2, 5]. Furthermore, expansion of the CD4⁺CD28^{null} T cell subset correlated directly with an increased frequency of extra-articular involvement like rheumatoid vasculitis and the clinical severity of the disease [3, 4]. These results suggest that the expansion of CD4⁺CD28^{null} T cells does not represent just an epiphenomenon but could play a critical role in the pathogenesis of the disease. Interestingly, in contrast to classical CD4⁺CD28⁺ T lymphocytes, CD4⁺CD28^{null} T cells express CX3CR1 which provides signals that support the activation of these cells in vitro [6]. Compared to CD28 T lymphocytes, CD4⁺CD28^{null} T cells isolated from patients with RA induced a significantly higher proliferation of synovial cell lines established from rheumatoid synovium [7]. This effect was mediated through CX3CR1 and amplified by the production of TNF- α from CD4⁺CD28^{null} T cells. Excessive proliferation of synovial fibroblasts causes bone and cartilage destruction in RA. Therefore, these in vitro studies suggest that CD28^{null} T cells promote bone and cartilage destruction in RA.

Recent discoveries highlighting the role of inflammation and immune responses in atherosclerosis have led to a better understanding of the mechanisms underlying CAD development and novel clinical approaches to this disease [8–10].

T lymphocytes, the main “soldiers” of the adaptive immune system, are consistently found in atherosclerotic lesions and contribute to their growth and activity [11]. At the early stages of atherosclerosis, T cells are involved in the initiation and progression of the disease, while at more advanced stages they contribute to the destabilization of atherosclerotic lesions [12, 13].

Most of the T cells present in atherosclerotic plaques are CD4 helper T lymphocytes [14]. Detailed analysis of CD4 T cells in CAD revealed the expansion of an unusual subset of

lymphocytes, characterized by the lack of the CD28 co-stimulatory receptor and therefore named CD4⁺CD28^{null} T cells [15]. Recent studies suggested that CD4⁺CD28^{null} T cells mediate plaque instability and recurrence of acute coronary events [16].

Complications of rheumatoid arthritis may begin to develop within months of presentation; therefore, early referral to or consultation with a rheumatologist for initiation of treatment with disease-modifying anti-rheumatic drugs is recommended, including tumor necrosis factor inhibitors, non-steroidal anti-inflammatory drugs, and corticosteroids, non-pharmacologic modalities are useful as well. Patients who do not respond well to a single disease-modifying drug may be candidates for combination therapy [17]. Moreover, advances in clinical research suggest that the inflammatory and cytotoxic function of CD4⁺CD28^{null} T cells can be inhibited by blocking OX40 and 4 to 1BB co-stimulatory receptors. Modulation of co-stimulatory receptors may allow specific targeting of this cell subset and may improve survival of ACS patients [18].

Patients and Methods

Study Population

42 patients (35 women, 7 men, aged 25–55 years, mean 41 ± 10 years) with rheumatoid arthritis diagnosed according to the criteria of the American College of Rheumatology were included in the study. Disease duration was 2–13 years (mean 6 ± 2.5 years). Patients were recruited from the outpatient clinic of the Department of Rheumatology and Physical Medicine, Kasr El Aini University Hospital, Cairo, Egypt.

The control group consisted of 10 healthy subjects (8 women and 2 men, aged 29–65 years, mean age 51 years).

Patient consent was obtained using the form approved by the Ethics Committee of the National Research Center.

Laboratory and Clinical Examination

Subjects underwent routine Hb, ESR, CRP, and lipid profile blood analysis in addition to antinuclear antibodies. In all patients, X-rays were made of the chest, hands, feet, and, when

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Table 1. Clinical characteristics of patients with RA subdivided into groups.

	Group 1	Group 2	Group 3	p
n	10	12	20	–
Age of onset (years)	39.6 ± 11.4	43 ± 8.2	40.8 ± 10.8	0.727
HB (g/dl)	12.7 ± 0.7	12.6 ± 0.5	11.8 ± 1.2	0.023*
Total cholesterol (mg/dl)	204.8 ± 41	204 ± 39.3	194.4 ± 35.4	0.699
HDL (mg/dl)	39.7 ± 1.1	38.9 ± 2.1	38.3 ± 3.2	0.389
LDL (mg/dl)	128.1 ± 39	134.3 ± 37.7	116.6 ± 35.2	0.404
TG (mg/dl)	167.5 ± 54.1	156.2 ± 35.3	194.8 ± 35.1	0.031*
ESR (1 st hour)	23.3 ± 11	24.4 ± 7.3	40.8 ± 24.6	0.019*
Disease duration (years)	5.6 ± 2.1	6.1 ± 1.5	6.3 ± 3.2	0.759
CD4 ⁺ CD28 [–] < 15 %	11.7 ± 2	11.3 ± 2	10.1 ± 3.2	0.000*
CD4 ⁺ CD28 [–] ≥ 15 %	21.8 ± 3	29.6 ± 5.4	44.5 ± 9.8	0.000*

Data are expressed as mean ± standard deviation.

* p < 0.05 means significant result.

Table 2. Comparison of the expansion of CD4⁺CD28⁺ and CD4⁺CD28^{null} T lymphocytes between RA patient groups and healthy controls

	Group 1	Group 2	Group 3	Controls	p
n	10	12	20	10	–
CD4 ⁺ CD28 [–] < 15 %	11.7 ± 2	11.3 ± 2	10.1 ± 3.2	10.5 ± 2.9	0.903
CD4 ⁺ CD28 [–] ≥ 15 %	21.8 ± 3	29.6 ± 5.4	44.5 ± 9.8	15.6 ± 0.56	0.000*

Data are expressed as mean ± standard deviation.

* p < 0.05 means significant result.

required, other joints. Evaluation included physical examinations with attention to patterns of joint involvement, presence of nodules, and other extra-articular features such as anemia, secondary Sjogren's, pulmonary affection, dryness of eyes, coronary artery disease, and laboratory features such as erythrocyte sedimentation rate and rheumatoid factor. To examine whether the presence of large numbers of CD4⁺CD28^{null} T cells in patients with RA is predictive of disease manifestation, the patients were allocated according to their disease pattern as follows:

- Group 1 included patients (n = 10; mean age 39.6 years, mean disease duration 5.6 years) with RA limited to joints (< 6 swollen joints) and without extra-articular manifestations.
- Group 2 included patients (n = 12; mean age 51.4 years, mean disease duration 6.1 years) with advanced joint manifestations (> 6 swollen joints) and with radiologically diagnosed erosions, without subcutaneous nodulosis or extra-articular manifestations.
- Group 3 comprised patients (n = 20; mean age 40.8 years, mean disease duration 6.3 years) with extra-articular manifestations; included 8 patients with nodulosis, 3 with anemia, 6 with secondary Sjogren's, 6 with pulmonary affection, 3 with dryness of eyes, 1 with scleritis, 8 with CAD, overlapping between extra-articular manifestations was observed.

Flow Cytometry

Peripheral blood mononuclear cells were obtained by Ficoll gradient centrifugation. The cells (300,000–500,000) were stained with fluorescein isothiocyanate- (FITC-) conjugated anti-CD4 (MCA1267F) and phycoerythrin- (PE-) conjugated anti-CD28(MCA709PE) monoclonal antibodies (AbD-Serotec-MorphoSys).

Analysis of peripheral blood mononuclear cells was performed using a FACSort flow cytometer (fluorescent-activated cell sorter). The T lymphocytes were excited with 488-nm light from a 15 mW argon ion laser. Logarithmic green and

red fluorescences of FITC and PE were measured through 530/30 nm and 585/42 nm bandpass filters, respectively.

Intact T lymphocytes were identified by their size as assessed by their logarithmic amplification of the FSC and SSC signals. CD4⁺CD28[–] T lymphocyte regions are selected by plotting CD4 versus CD28.

The number of cells expressing CD4- or CD28-emitting fluorescence signals were multiplied in PMT, then the computer analysed the data and results were expressed as a percentage of cells.

Two-color flow cytometric immunostaining of subjects' peripheral blood showed that bivariate FSC/SSC efficiently distinguished intact T lymphocytes and the mixture of lymphocytes and platelets that scattered within the same FSC/SSC gate. The purity as well as the proportion of T lymphocytes in this gate were identified further using FITC/PE fluorochrome-conjugated monoclonal antibodies to CD4 and CD28.

Then the percentage of circulating T lymphocytes from each group was identified. The fraction of cells within the CD4⁺ population that was CD28[–] was calculated by gating on the CD4⁺CD28⁺ and CD4⁺CD28[–] populations.

Statistical Analysis

Computer software package SPSS 13 was used in the analysis for quantitative variables; mean (as a measure of central tendency), standard deviation, and range (as measures of variability) were presented.

The chi square test was used to assess differences between qualitative variables while independent sample t-test and ANOVA (analysis of variances) tests were used for quantitative variables.

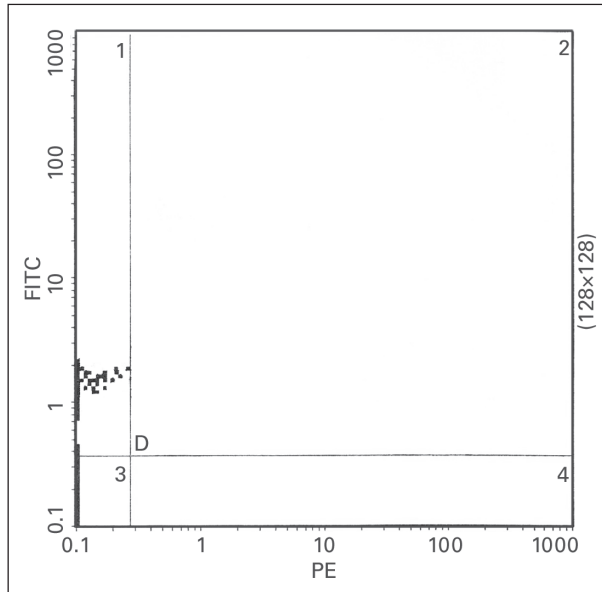
To estimate association between expansion of CD4⁺CD28[–] T lymphocytes and other variables, Pearson's test was done; correlation coefficient and P value were presented.

For T lymphocytes percentages and all other values among the groups of subjects, the threshold for statistical significance for all comparisons was chosen as p ≤ 0.05.

Table 3. Number and percentage of CD4⁺CD28⁺ and CD4⁺CD28^{null} T lymphocytes between RA patient groups and healthy controls.

	CD4 ⁺ CD28 ⁺ < 15 %			CD4 ⁺ CD28 ⁺ ≥ 15 %		
	n	%	Mean ± SD	n	%	Mean ± SD
Cases	10	23.8	11.12 ± 2.2	32	76.2	36.09 ± 12.2
Controls	8	80	10.5 ± 2.9	2	20	15.6 ± 0.5

Data are expressed as percentage, mean, and standard deviation.

**Figure 1.** Flow cytometry dot plot showing CD4⁺CD28^{null} cells gated on a CD4⁺ T cell population in RA patients.

Results

The results of the present study confirmed that the number of CD4⁺ cells lacking the CD28 molecule was not correlated with age, cholesterol, LDL, HDL, and disease duration in RA patients (Table 1). The frequency of CD4⁺CD28^{null} T cells (Table 2) was significantly higher in RA (group 1: 21.8 ± 3, group 2: 29.6 ± 5.4, group 3: 44.5 ± 9.8) than in control subjects (15.6 ± 0.56) with a significant p value (0.000). RA patients with a percentage of CD4⁺CD28^{null} T cells subset > 15 % (90th percentile of the distribution in the normal population) were defined as having expansion of the

CD4⁺CD28^{null} T cells, the number of RA patients with this expansion is 32 patients (76.19 %; Table 3, Figure 1). Moreover, this study shows that patients with CD4⁺CD28^{null} T cell expansion were characterized by a higher frequency of extra-articular manifestations (44.5 ± 9.8 vs 26.5 ± 6; p = 0.000), and a slight non-significant increase toward a more erosive disease (37.2 ± 12.3 vs 34.8 ± 12.3; p = 0.588, ns). Moreover, incidence of CAD was higher in RA patients with evidence of CD4⁺CD28^{null} cell T cell expansion (47.9 ± 6.7 vs 32.7 ± 11.3; p = 0.000). Patients having negative RF showed more CD4⁺CD28^{null} cell T cell expansion than those with positive RF (39.9 ± 13 vs 30.4 ± 8.5; p = 0.019; Table 4).

Discussion

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease. It is associated with increased mortality, mostly due to an excess of cardiovascular disease. Indeed, the average lifespan of individuals with RA is shortened by 3–18 years. Even after adjustment for traditional cardiovascular risk factors, there is a higher rate of cardiovascular events in subjects with RA than in healthy subjects, which suggests that additional mechanisms are responsible for the excess cardiovascular risk observed in RA [19].

Coronary artery disease (CAD), a manifestation of atherosclerosis, continues to be the most common cause of death in the developed world. Atherosclerosis is predominantly an inflammatory process that involves both the innate and adaptive arms of the immune system [20].

CD4⁺CD28^{null} T cells are expanded in the peripheral blood of patients with unstable angina (UA) and infiltrate unstable coronary plaques, where they undergo clonal expansion, probably triggered by specific antigens [16].

CD4⁺CD28^{null} T cells differ from conventional CD4⁺CD28⁺ helper T lymphocytes in both phenotype and function. CD4⁺CD28^{null} T cells are terminally differentiated and have pro-inflammatory functions characterized by the production of high levels of interferon-γ, tumor necrosis factor-α, and IL-2. In addition, CD4⁺CD28^{null} T cells are cytotoxic and effectively kill endothelial cells in vitro. This is mediated by cytolytic enzymes, such as perforin, granzyme A, and granzyme B expressed by CD4⁺CD28^{null} T cells [6].

Disease-associated expansions of these CD4⁺CD28^{null} T cells have been reported in inflammatory disorders such as rheumatoid arthritis, Wegener's granulomatosis, multiple sclerosis, and ankylosing spondylitis and in chronic infections [21].

Table 4. Clinical characteristics of patients with RA divided by the presence or absence of peripheral blood CD4⁺CD28^{null} T cell expansion.

		CD4 ⁺ CD28 ⁺ < 15 %	p	CD4 ⁺ CD28 ⁺ ≥ 15 %	p
RF	Seronegative	10.8 ± 2.3	0.406	39.9 ± 13	0.019*
	Seropositive	12.4 ± 2.5		30.4 ± 8.5	
ANA	Seronegative	9.4 ± 2.7	0.123	36.6 ± 12.2	0.905
	Seropositive	11.8 ± 1.7		35.9 ± 12.4	
CRP	Negative	11 ± 0.4	0.921	34.2 ± 12.1	0.230
	Positive	11.2 ± 2.7		39.7 ± 12.2	
Bony erosion	Present	11.3 ± 1.7	0.747	37.2 ± 12.3	0.588
	Absent	10.7 ± 3.7		34.8 ± 12.3	
Extra-articular manifestation	Present	10.1 ± 3.2	0.385	44.5 ± 9.8	0.000*
	Absent	11.5 ± 1.8		26.5 ± 6	
CAD	Present	6.5 ± 0.1	0.021*	47.9 ± 6.7	0.000*
	Absent	11.6 ± 1.6		32.7 ± 11.3	

Data are expressed as mean ± standard deviation.

* p < 0.05 means significant result.

One of the best characterized autoimmune diseases associated with increased frequencies of CD4⁺CD28^{null} T cells is rheumatoid arthritis (RA). Expansion of the CD4⁺CD28^{null} T cell subset correlated directly with an increased frequency of extra-articular involvement like rheumatoid vasculitis, and the clinical severity of the disease. These results suggest that the expansion of CD4⁺CD28^{null} T cells does not represent just an epiphenomenon but could have a critical role in the pathogenesis of the disease [5].

The present study aimed to detect the percentage of expansion of CD4⁺CD28^{null} T lymphocytes in the peripheral blood of patients with rheumatoid arthritis which could be a good marker correlating with extra-articular manifestations, CAD, and extent of joint involvement.

In this study, we provided evidence of the presence of circulating CD4⁺CD28^{null} T lymphocytes in rheumatoid arthritis patients as well as normal blood samples; their percentage in the patient groups showed a higher range when compared to the control group. This may be explained by the fact that clonal expansion of CD4⁺CD28^{null} T cells is a characteristic finding in patients with rheumatoid arthritis. Expanded CD4⁺ clonotypes are present in the peripheral blood, infiltrate into the joints, and persist for years. CD4⁺CD28^{null} T cells are oligoclonal lymphocytes that are rare in healthy individuals but are found in high percentages in patients with chronic inflammatory diseases [4].

Also this result coincides with Fasth et al [22], who reported that in the CD4⁺ T cell population the expression of NKR (NK-cell receptors) is primarily found in the CD4⁺CD28^{null} T cell subset. These T cells are frequently found in rheumatoid arthritis.

Among RA patients, the frequency of these CD4⁺CD28^{null} T cell lymphocytes was significantly higher than in controls. Nevertheless, the CD4⁺CD28^{null} T cell compartment differed depending on extra-articular manifestations and joint involvement. The lowest frequency of CD4⁺CD28^{null} T cells was in RA patients with limited joint manifestations. Significantly higher numbers of CD28-lacking lymphocytes were present in patients with extra-articular manifestations, especially patients with CAD. This may be explained by the observation that one of the consequences of dysregulated T cell homeostasis is the emergence of large clonal CD4⁺CD28^{null} T cell populations that are autoreactive and cytotoxic and infiltrate synovial tissue. The highest frequency of CD4⁺CD28^{null} T cells is found in severe RA, particularly in patients with rheumatoid vasculitis. When the inflammatory process in RA spreads to extra-articular sites, such as mid-size arteries and capillaries, morbidity and mortality are clearly increased [23]. In addition, in acute and most severe manifestations of CAD, ie, myocardial infarction and sudden death result from the rupture of a plaque which causes the formation of a thrombus and sudden occlusion of the artery. The presence of activated immune cells, thinning of the fibrous cap, disruption of the collagen matrix, and apoptosis of smooth muscle cells have been implicated in the destabilization of atherosclerotic lesions leading to plaque rupture. Among immune cells, macrophages have a pivotal role in plaque rupture through the release of extracellular matrix-degrading enzymes like metalloproteinase (MMPs). CD4⁺CD28^{null} T cells have been shown to accumulate preferentially in unstable atherosclerotic plaques, therefore being well-located to induce plaque destabilization [24].

Also it was observed that patients with unstable angina (UA) and frequent recurrence of acute coronary events have a median frequency of CD28-lacking CD4⁺ cells about 4× higher than patients with a first-ever acute coronary event during 4 years of clinical observation, and about 9× higher than patients with chronic stable angina (CSA). Moreover,

on multivariate logistic regression analysis, CD4⁺CD28^{null} T cell frequency was an independent predictor of future acute coronary events [16].

Phenotypic and functional properties of CD28-lacking CD4⁺ cells may provide alternative explanations for a possible pathogenic role of this cell subset in favoring CV disease in RA. The expression of CD28 is regulated by a complex cytokine network. T cell activation in the presence of interleukin-12, a proinflammatory cytokine highly expressed in the inflammatory environment of a rheumatoid joint [25, 26], results in the restoration of CD28 gene transcription and cell surface appearance of a functional CD28 molecule on CD4⁺CD28^{null} cells. This may account for the finding that CD4⁺CD28^{null} cells partially regain expression of CD28 in rheumatoid synovium [2]. More interestingly, the up-regulatory action exerted by interleukin-12 on CD28 expression is contrasted by down-modulation induced by another proinflammatory cytokine, TNF- α , which plays a fundamental role in several chronic inflammatory conditions [27]. Thus the description of CD4⁺CD28^{null} T cells in vivo not only during normal aging [28, 29], in which elevated levels of TNF- α have been described [30], but also among patients with inflammatory diseases, including RA [31]. These CD4⁺CD28^{null} T cells have a proinflammatory activity. In particular, they produce large amounts of interferon- γ [32], a typical Th1-cytokine that is involved not only in rheumatoid synovitis but also in atherosclerosis development [15, 26, 33].

Among our RA patients, Hb levels were significantly lower in RA patients with extra-articular manifestations when compared with RA patients with limited joint manifestations and patients with advanced joint involvement.

Anemia is the most common extra-articular manifestation of RA, estimated to occur in 30–60 % of RA patients. There is evidence that patients who are anemic have more severe RA, and also have more affected joints and higher levels of functional disability and pain [34–36].

Both IFN- γ and TNF- α are known for their direct negative effects on erythropoiesis, and TNF- α can shorten erythrocyte half-life because of an increased phagocytosis by macrophages. The presence of an expanded CD4⁺CD28^{null} T cell population with an exceptionally high proinflammatory profile offers a pathogenetic concept for this association. The increased basal TNF- α production of CD4⁺CD28^{null} T cells compared with CD4⁺CD28⁺ cells may explain the effect of these cells on erythropoiesis [37].

This study suggests an association between seronegative RF, ESR, and extra-articular manifestations, especially CAD. Previous studies proved that particularly ESR are significantly associated with the risk of CVD in RA [38], on the other hand, Miguel et al [39] reported that there is no correlation between ESR and intima-media thickness (IMT) of the common carotid artery, an indicator of greater risk of developing cardiovascular events, also other studies showed that elevated RF is associated with CAD [40] opposing our results.

The present study confirmed the association between hypertriglyceridemia and patients suffering from extra-articular manifestations of rheumatoid arthritis.

The changes of lipoproteins during infection and inflammation such as hypertriglyceridemia, elevated triglyceride-rich lipoproteins, the appearance of small dense low-density lipoproteins, increased platelet-activating factor acetylhydrolase activity, and secretory phospholipase A2, sphingolipid-enriched lipoproteins, and decreased high-density lipoprotein (HDL) cholesterol are changes that could promote atherogenesis [41]. Moreover, recently it has been suggested that autoimmune disease contributes to the dyslipidemia process [42].

Conclusion

Elevated plasma ESR and triglycerides are independent risk factors for CAD in patients with rheumatoid arthritis. This finding implies an important relationship between inflammation and atherosclerosis and suggests that autoimmune processes may be involved in the development of CAD in patients with rheumatoid arthritis.

Based on this finding, we suggest that increased CD4⁺CD28^{null} expansion, ESR, and triglyceride levels together with seronegative RF can serve as markers to help identify patients with rheumatoid arthritis who are at risk of developing CAD, hoping that in the future these laboratory parameters could be included in an equation or ratio to predict early the possibility of CAD occurrence so as to prevent it.

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MTHFR C677T and A1298C Gene Polymorphisms, Hyperhomocysteinemia, and Intimal Medial Thickness as Risk Factors of End-Stage Renal Disease in Children on Hemodialysis

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Background: The methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism has been shown to be associated with cardiovascular disease and in patients with end-stage renal disease (ESRD). However, the relationship between MTHFR polymorphisms and cardiovascular disease (CVD) in patients on hemodialysis has not been examined. The aim of this study was to assess the association of polymorphisms of MTHFR gene with homocysteine (Hcy) and intimal medial thickness (IMT) in children on hemodialysis. **Methods:** We performed a case-control study comprising 55 pediatric patients with ESRD and 55 healthy children as controls. Plasma Hcy was measured in all the subjects and these subjects were genotyped for 2 MTHFR polymorphisms (C677T and A1298C). **Results:** We observed significantly higher Hcy levels in patients compared to controls. The frequency of MTHFR 1298CC genotype was significantly higher in ESRD children than in controls (21.82 % vs 5.45 %) and the frequency of the MTHFR 677TT genotype differs significantly between groups (18.18 % vs 0.00 %). The frequency of co-occurrence of MTHFR 677CT/1298AC and 677TT/1298CC was significantly higher in patients than controls and was associated with an increased risk of disease ($p < 0.05$). MTHFR 1298AC+CC genotypes were associated with higher Hcy levels. IMT was also significantly higher in patients with the 1298AC+CC genotypes ($p < 0.05$). **Conclusion:** A1298C polymorphism of MTHFR gene appears to be associated with the severity of carotid atherosclerosis and co-occurrence of MTHFR polymorphisms has a synergistic effect on increased risk of disease susceptibility. *J Clin Basic Cardiol* 2012; 15 (online): 7–12.

Key words: A1298C, C677T, MTHFR, end-stage renal disease, children, single nucleotide polymorphism

Introduction

Hemodialysis (HD) patients have a much higher overall mortality rate than the general population [1, 2]. Cardiovascular disease (CVD), a frequent complication in these patients, is a major cause of the mortality [3]. In this setting, important risk factors for cardiovascular disease include hypertension, diabetes mellitus, and increased oxidant stress [4]. Hyperhomocysteinemia has recently been identified as a predictor of atherosclerotic complications in the general population [5–8]. It is also frequent among patients with renal failure [9, 10]. Several recent studies showed that in dialysis patients, hyperhomocysteinemia was a risk factor for cardiovascular complications [10–12].

Serum and intracellular levels of homocysteine (Hcy) are regulated by remethylation to methionine or transsulfuration to cysteine [13]. The methyl donor in the vitamin B₁₂-dependent remethylation is 5-methyltetrahydrofolate generated from the reduction of 5,10-methylenetetrahydrofolate by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). Elevated plasma concentrations of total Hcy (tHcy) result chiefly from genetic defects in the enzymes involved in homocysteine metabolism or from nutritional deficiency of the vitamin cofactors [14]. Because renal uptake and metabolism normally account for about 70 % of Hcy elimination from plasma [10, 15], impaired renal function also results in hyperhomocysteinemia [9, 10]. Hcy causes endothelial cell dysfunction and injury via production of potent reactive oxygen species during its autooxidation [14, 16]. This metabolite is thrombogenic, as it increases thromboxane formation, antagonizes nitric oxide, enhances platelet aggregation, and inhibits protein C and thrombomodulin [14, 16]. Hcy also is a potent mitogen for vascular smooth muscle cells [17, 18]. Therefore, in end-stage renal disease, elevated plasma Hcy concentrations could contribute to the high prevalence of cardiovascular disease and the increased

mortality rate [10–12, 19]. Hyperhomocysteinemia at an earlier stage could also accelerate progression of chronic renal disease [19].

Two polymorphisms, MTHFR C677T and MTHFR A1298C, decrease MTHFR activity and tend to increase Hcy levels [20]. A common C-to-T mutation at nucleotide position 677 (C677T) has been identified in the gene coding for MTHFR, which is involved in the remethylation of Hcy [20]. The C677T mutation causes a valine for alanine substitution, which decreases MTHFR activity and tends to elevate plasma concentrations of tHcy in individuals who are homozygous for the mutation (TT genotype) [20]. This genotype also has a reported association with coronary heart disease [21, 22], but this remains controversial [23]. In subjects with normal renal function, the TT genotype causes only a 25-% increase in plasma tHcy compared to subjects with other genotypes [23], but in patients with end-stage renal disease undergoing maintenance dialysis, the TT genotype causes a 40–100-% increase in plasma tHcy [19, 24, 25] compared to other genotypes that already have 2–3 times higher concentrations of tHcy than normal subjects. The compound heterozygosity of MTHFR A1298C polymorphism with other MTHFR polymorphisms has been associated with either increased or decreased Hcy levels [20].

In this study, we examined the association of these 2 polymorphisms of the MTHFR gene, ie, C677T and A1298C, with ESRD in children with hyperhomocysteinemia and intimal medial thickness (TMT).

Methods

55 Egyptian pediatric patients with end-stage renal disease on hemodialysis were included in the study. HD children were selected from the hemodialysis unit of the Center of Pediatric Nephrology and Transplantation (CPNT), Children's Hospital, Cairo University. The study was performed from Janu-

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Table 1. Demographic profile of the study population

	Maintenance hemodialysis	Controls
n	55	55
Age (years)	10.62 ± 3.49	10.7 ± 4.51
Gender		
Male	29 (52.73 %)	30 (54.55 %)
Female	26 (47.27 %)	25 (45.45 %)
BMI (kg/m ²)	18.89 ± 3.00	20.60 ± 1.44
SBP (mmHg)	126.13 ± 17.36*	94.54 ± 9.70
Indexed SBP	1.03 ± 0.14**	0.73 ± 0.05
DBP (mmHg)	85.13 ± 13.76*	62.55 ± 9.10
Indexed DBP	1.00 ± 0.10**	0.75 ± 0.05
eGFR (ml/min/1.73m ²)	11.20 ± 3.35**	87 ± 8.8
Dialysis (years)	2.74 ± 1.58	–
Kt/V	1.68 ± 0.45	–
Creatinine (mmol/l)	567.5 ± 128.2*	63.65 ± 28.28
Predialysis urea (mmol/l)	25.19 ± 7.00*	2.77 ± 0.90
Total cholesterol (mg/dl)	193.04 ± 50.37*	160.31 ± 17.75
Albumin (g/dl)	3.68.35*	4.93.39
Calcium (mmol/l)	2.48 ± 0.10*	2.57 ± 0.42
Serum phosphate (mmol/l)	1.26 ± 0.15**	1.63 ± 1.10
Hemoglobin (g/dl)	10.53 ± 1.83*	14.20 ± 1.50
Homocysteine (μmol/l)	35.21 ± 37.24*	16.11 ± 14.50
CIT (mm)	0.49 ± 0.09**	0.40 ± 0.03

BMI: Body Mass Index; SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; Kt/V: adequacy of hemodialysis; CIT: carotid intimal thickness.

*p < 0.05 or **p < 0.01 vs controls and HD

ary 2011 to June 2011. The causes of renal failure were: hereditary nephropathies (n = 21), obstructive uropathies (n = 6), neurogenic bladder (n = 4), glomerulopathy (n = 3), renal hypoplasia or dysplasia (n = 2), and unknown causes (n = 19). Inclusion criteria for HD patients included a constantly elevated serum creatinine level above the normal range (ranging from 3.4–15.8 mg/dl) and dialysis for at least 6 months. They were treated with hemodialysis for 3–4 h 3 times weekly with a polysulfone membrane using bicarbona-

te-buffered dialysate. Duration of hemodialysis was 2.82 ± 1.37 years. 31 HD patients were taking anti-hypertensive treatment. The following classes of drugs were employed: α-adrenoceptor antagonists in one HD patient, betablockers in 9, ACE inhibitors in 17, and calcium channel blockers in 4.

To control for differences in age and body size, blood pressures were indexed to the age-, gender-, and height-specific 95th percentile for each subject (measured systolic [SBP] or diastolic blood pressure [DBP] was divided by the age-, gender-, and height-specific 95th percentile). Hypertension was defined as indexed SBP or DBP ≥ 1.0. None of HD patients had cardiovascular events on the basis of examination and detailed clinical history.

All control subjects (n = 55) were healthy with no clinical signs of vascular or renal disease and no family history of renal disease as assessed by medical history and clinical examination, as well as a lack of medications taken at the time of the study. Healthy control subjects were selected to be matched for age and gender to the patient groups, as well as within the same BMI limits. They were collected from the pediatric clinic (apart from the medical services unit) of the National Research Centre (NRC) which is one of the biggest research centers in Egypt. Informed consent for genetic studies was obtained from the parents of all participants. The protocol of the study was read and approved by the ethics committee of the NRC in Egypt.

Biochemical Markers

Venous blood samples were collected in the morning after an overnight fast on a midweek dialysis day before the dialysis session. Three ml of venous blood was collected in EDTA vials for the extraction of genomic DNA. Pre- and post-dialysis kidney function tests were determined by standard laboratory methods. Hemoglobin, albumin, serum calcium, serum phosphorous, blood urea nitrogen, and creatinine were measured for all patients and controls using an automatic biochemistry analyzer (Olympus America Inc, Center Valley, PA, USA).

Quantitative Determination of Homocysteine Levels in Serum

The Diazyme homocysteine enzymatic assay is based on a novel assay principle that assesses the co-substrate conversion product instead of assessing the co-substrate itself. The concentration of homocysteine in the sample is indirectly proportional to the amount of NADH converted to NAD⁺.

Table 2. MTHFR polymorphisms: genotype and allele frequency in ESRD children and controls

	HD	Controls	OR	95-% CI	p
n	55	55	–	–	–
Genotypes					
677CC	28 (50.91 %)	35 (63.64 %)	0.97	0.72–1.32	0.88
677CT	17 (30.91 %)	20 (36.36 %)	1.04	0.62–1.73	0.87
677TT	10 (18.18 %)*	0 (00.00 %)	0.46	0.32–0.65	0.002
1298AA	15 (27.27 %)	44 (80.00 %)	0.32	0.20–0.52	0.52
1298AC	28 (50.91 %)	8 (14.55 %)	0.87	0.19–3.88	0.86
1298CC	12 (21.82 %)**	3 (5.45 %)	0.09	0.03–0.26	0.0001
Alleles					
677C	73 (66.36 %)	90 (81.82 %)	0.70	0.54–0.92	0.16
677T	37 (33.64 %)	20 (18.18 %)	1.52	1.04–2.21	0.17
1298A	58 (52.73 %)	96 (87.27 %)	2.92	1.81–4.75	0.35
1298C	52 (47.27 %)**	14 (12.73 %)	0.16	0.08–0.32	0.0001

OR: odds ratio; CI: confidence interval. *P < 0.05 or **p < 0.001.

The kit was supplied by Diazyme laboratories (USA), catalog # D Z568A [26].

Intima-Media Thickness Measurement

Carotid artery ultrasound was carried out to measure (IMT) using the Vivid 3 (Norway) ultrasonography machine with 3- and 7-MHz linear array transducers. Imaging protocols were followed as described by Sidhu et al [27].

Genotyping of MTHFR Gene Variants

DNA was extracted using the spin column QIAamp blood kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) amplification and enzymatic digestion of the products were done. For the methylene tetrahydrofolate reductase C677T polymorphism genomic DNA of 0.5–2 mg was amplified each with a forward primer '50 TGAA-GGAGAAGGTGTCTGC GGA-3' and a reverse primer '50 AGGACGGTGCGGT GAGAGTG-3' (supplied by Biosynthesis). PCR was performed with a Biometra thermoblock under standard conditions, for 40 cycles and with 72 °C as annealing temperature. After amplification, the PCR product was digested with restriction endonuclease *HinfI* and electrophoresed in a 4–% agarose gel. With the enzyme *HinfI* (Fermentas, Lithuania), the respective genotypes were defined as:

1. Wild type (677CC; indicating the absence of the restriction site)
2. Heterozygote (677CT) produced 198, 175, and 23 base pair fragments
3. Homozygous mutants (677TT) produced 175 and 23 base pair fragments

For the methylene tetrahydrofolate reductase A1298 C polymorphism genomic DNA of 0.5–2 mg was amplified each with a forward primer '50 CTTTGGGGAGCTGAA-GGAC TACTAC-3' and a reverse primer '50 CACTTTGT GACCATTCCGGTTTG 3' (supplied by Biosynthesis). PCR was performed with a Biometra thermoblock for 35 cycles and with 72 °C as annealing temperature. After amplification, the PCR product was digested with restriction endonuclease *MboII* and electrophoresed in a 3–% agarose gel with the enzyme *MboII* (Fermentas, Lithuania). The respective genotypes were defined as:

1. Wild type (1298AA) if they produced 5 fragments of 56, 31, 30, 28, and 18 base pair
2. Heterozygote (1298AC) if they produced 6 fragments of 84, 56, 31, 30, 28, and 18 base pair

3. Homozygous mutants (1298CC) if they produced 4 fragments of 84, 31, 30, and 18 base pair.

The major visible bands were those at 84 and 56 base pair [28].

Statistical Analysis

The Statistical Package for Social Science (SPSS) program, version 16.0, was used for data analysis. Data were summarized as mean $\pm$ SD, range, or percentage. Histograms and normality plots were used for evaluating the normality of data. For those data with skewed distribution, log transformation was performed before a t-test.

A sample size of 20 will give us approximately 80 % power ($\alpha = 0.05$, 2-tail) to reject the null hypothesis of zero correlation. We used power calculations performed by the Power and Precision program (Biostat) to determine the number of chromosomes required to detect a significant difference between the polymorphism frequency in the reference population and the expected frequency. Data were evaluated between the experimental groups by independent samples t-test. Allele and genotypic frequencies for MTHFR C677T and MTHFR A1298C alleles were calculated with the gene-counting method. Hardy-Weinberg equilibrium was tested by using the Pearson Chi-square (χ^2) test. A 2×2 contingency table was used for test of the differences of allele frequencies between cases and controls. Odds ratios (OR) with 95-% confidence intervals (CI) were estimated for the effects of high-risk alleles. Clinical characteristics of ESRD children with different MTHFR C677T and MTHFR A1298C genotypes were compared using an independent t-test. $P < 0.05$ was considered statistically significant.

Results

Anthropometric, clinical, and biochemical parameters in controls and ESRD children are shown in Table 1. Hcy levels were significantly higher in the patient group compared to controls ($p < 0.05$).

Prevalence of MTHFR Genotypes

The genotype and allele frequencies for the MTHFR C677T and A1298C polymorphisms are shown in Table 2. The population was in Hardy-Weinberg equilibrium (HWE) for MTHFR C677T and MTHFR A1298C genotypes. The homozygous mutant of MTHFR 677 TT genotype was significantly higher in our study population ($p < 0.05$). The fre-

Table 3. Association of various parameters with different MTHFR genotypes in ESRD children.

	677CC	677CT+TT	1298AA	1298AC+CC
n	28	27	15	40
BMI (kg/m ²)	18.97 $\pm$ 2.77	18.75 $\pm$ 3.52	18.53 $\pm$ 3.01	17.16 $\pm$ 3.06
SBP (mmHg)	116.32 $\pm$ 18.62	124.23 $\pm$ 19.01	119.68 $\pm$ 18.88	123.57 $\pm$ 19.85
DBP (mmHg)	76.94 $\pm$ 13.59	85.00 $\pm$ 15.02*	79.57 $\pm$ 13.49	85.32 $\pm$ 14.25*
Creatinine (mmol/l)	442.00 $\pm$ 174.15	571.95 $\pm$ 140.56*	476.48 $\pm$ 165.31	581.67 $\pm$ 148.51
Urea (mmol/l)	24.88 $\pm$ 7.40	24.34 $\pm$ 8.08	25.17 $\pm$ 7.28	25.39 $\pm$ 4.37
Albumin (g/dl)	3.67 $\pm$ 0.28	3.44 $\pm$ 0.27	3.73 $\pm$ 0.27	3.55 $\pm$ 0.29
Calcium (mmol/l)	2.32 $\pm$ 0.42	2.36 $\pm$ 0.31	2.41 $\pm$ 0.44	2.38 $\pm$ 0.31
Phosphate (mmol/l)	1.63 $\pm$ 1.11	1.51 $\pm$ 1.22	1.02 $\pm$ 1.08	1.54 $\pm$ 1.25
Cholesterol (mg/dl)	188.78 $\pm$ 55.03	198.11 $\pm$ 43.07	181.55 $\pm$ 51.09	187.77 $\pm$ 53.25
CIT (mm)	0.49 $\pm$ 0.10	0.49 $\pm$ 0.09	0.53 $\pm$ 0.15	0.48 $\pm$ 0.07*
Homocysteine (μ mol/l)	38.25 $\pm$ 36.56	42.31 $\pm$ 43.02	34.66 $\pm$ 24.85	42.34 $\pm$ 30.73*

All values in mean $\pm$ SD (Standard deviation), * $p < 0.05$ was considered significant.

quencies of MTHFR 1298CC genotype and mutant allele C were significantly higher in patients compared to controls ($p < 0.001$) and were associated with an increased risk of ESRD.

The association of various parameters (biochemical and clinical) with MTHFR genotypes is shown in Table 3. Increased serum creatinine levels were observed in MTHFR 677CT+TT as compared to 677CC genotype ($p < 0.05$). Mean IMT in patients with 1298AC+CC genotypes was significantly higher compared to 1298AA ($p < 0.05$). No significant change was observed in mean IMT between MTHFR 677CT+TT and 677CC genotypes. No significant association of mean plasma Hcy levels was seen between the MTHFR C677T genotype. However, patients with 1298AC+CC genotype had significantly higher levels of Hcy ($p < 0.05$) compared to the 1298AA genotype.

MTHFR C677T and A1298C Polymorphisms

The cross-tabulation of genotype combinations of MTHFR C677T and A1298C are given in Table 4. The frequency of co-occurrence of MTHFR 677CT/1298AC and 677TT/1298CC was significantly higher in patients than controls and was associated with an increased disease risk ($p < 0.05$).

Discussion

Hyperhomocysteinemia has been commonly observed in patients with chronic renal failure, owing to both altered metabolism and reduced renal excretion [29]. Significantly increased Hcy levels in ESRD patients were also observed. An association between mild hyperhomocysteinemia and occlusive vascular disease has been reported although the findings are inconsistent [30–32]. Many studies have shown that folic acid can reduce elevated homocysteine levels [33, 34] but has not yet been shown to improve the progression of atheroma or cardiovascular outcome in chronic renal failure (CRF). A recent study on the effect of high-dose folic acid on the progression of atherosclerosis and cardiovascular events in patients with CRF has shown that high-dose folic acid does not slow atheroma progression or improve either cardiovascular morbidity or mortality in patients with CRF [35]. Further interventional studies are needed to see if folate/vitamin supplementation could have beneficial effects in patients with chronic renal failure.

Significantly higher serum PO₄ and calcium levels were observed in ESRD patients in our study. Similar observations have been made by others in patients with chronic renal failure [36]. The increased levels of Ca/PO₄ have been suggested to contribute to arterial calcium deposition in these patients. We observed increased carotid IMT in ESRD children, which further supports a possible association of high serum Ca/PO₄ with cardiovascular disease in these patients. In an independent sample *t* analysis, serum calcium phosphate levels were similar in all MTHFR genotypes, however, IMT showed association with MTHFR 1298AC+CC geno-

types. Thus these observations do not suggest a possible association between MTHFR genotypes and arterial calcifications, but further studies are needed with larger sample size to confirm these findings.

Polymorphisms in MTHFR gene have been shown to be associated with increased homocysteine levels and possibly an effect on cardiovascular mortality. Patients with chronic kidney diseases who are on dialysis are known to have increased plasma Hcy concentrations. However, there is inconsistent evidence of any association between MTHFR genotypes and ESRD. We studied the respective frequencies and co-occurrence of 2 functional MTHFR polymorphisms (C677T and A1298C), associated with plasma Hcy concentrations in ESRD children. We found that the homozygous mutant of MTHFR677 TT genotype was significantly higher in our study population and is associated with an increased disease risk; however, the highest disease risk was observed with the 1298CC genotype. The MTHFR C677T polymorphism has been inconsistently shown to be associated with chronic kidney diseases and renal failure in different ethnic populations. Previous studies in many ethnic groups including Chinese [37], Polish [38, 39], and Japanese [40] have demonstrated that MTHFR C677T may be a risk factor for renal diseases [41]. However, others failed to find such an association [42].

The MTHFR A1298C polymorphism is associated with decreased enzyme activity and could result in increased Hcy levels, suggesting a possible role in the pathogenesis of chronic renal failure [43]. The homozygous wild genotype (AA1298) has been reported to be protective in patients with diabetic nephropathy in the presence of low folate levels [44], however, others have not found any association of A1298C genotypes alone or in combination with plasma Hcy levels in hemodialysis patients [45].

The frequency of the variant C allele of 1298 gene was significantly higher in our pediatric population with ESRD. Fung et al [46] reported that the MTHFR-coding polymorphism at A1298C is associated with renal decline in African-Americans with hypertensive nephrosclerosis. We also observed significantly higher Hcy levels in patients with AC+CC genotypes. Thus, a higher frequency of CC genotype present in our population makes it extremely relevant in terms of its potential impact on ESRD in our pediatric population.

Intimal thickening is an adaptive process of the arterial wall, presumably related to atherogenesis and increased IMT of the carotid arteries has been considered a useful marker of atherosclerosis and cardiovascular complications [27, 47, 48]. We found that patients with 1298AC+CC genotypes had significantly higher IMT as compared to 1298AA genotype, which suggests that these genotypes may be predisposing these patients to higher risk of CVD.

Co-occurrence of 1298CC with 677CC genotype has also been shown to be associated with an increased cardiovascular disease risk in hemodialysis patients [44]. Koupepidou et al

Table 4. Combined genotype frequencies of MTHFR C677T and A1298C in pediatric patients and controls, n (%).

	Patients			Controls		
	55			55		
n _{tot}	MTHFR677CC	MTHFR677CT	MTHFR677TT	MTHFR677CC	MTHFR677 CT	MTHFR677TT
MTHFR1298AA	10 (7.27)	2 (20)	3 (3.64)	30 (54.55)	14 (21.82)	0 (0.00)
MTHFR1298AC	8 (21.82)	14 (27.27)*	6 (5.45)*	5 (18.18)	3 (3.63)	0 (0.00)
MTHFR1298CC	10 (7.27)	1 (3.64)	1 (3.64)*	0 (0.00)	3 (1.82)	0 (0.00)

* $p < 0.05$

observed that essential hypertensive patients with 677CT/1298AC genotypes were predisposed to develop hypertensive nephrosclerosis and chronic renal failure [49]. Födingen et al demonstrated that combined the MTHFR677TT/1298AA genotype was a predictor of total Hcy plasma levels in dialysis patients [43]. Thus, the increased risk with these genotypes may be due to their influence on Hcy levels [50]. We found that co-occurrence of MTHFR 677CT/1298AC and 677TT/1298CC was significantly higher in patients than in controls and was associated with an increased disease risk.

The present study has certain limitations. The source pediatric population of the study was an Egyptian population only, which renders generalizability to the other ethnic groups uncertain. Future studies are required to analyze the various MTHFR genotypes with disease prevalence in different ethnic pediatric populations.

Conclusion

Our results suggest that the presence of mutant alleles 1298C and 677T confers an increased risk for ESRD in children and a co-occurrence of these polymorphisms has a synergistic effect on increased risk of disease susceptibility.

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Conflict of Interest

The authors declare that they have no competing interests.

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Evaluation of Prognostic Stratification of Patients with Chronic Heart Failure by Recovery Cardiopulmonary Indices Compared to Cardiopulmonary Exercise Indices

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Background: Cardiopulmonary exercise testing (CPET) with monitoring of gas exchange parameters provides useful information on exercise capacity and prognosis in congestive heart failure (CHF). Little is known about prognostic importance of recovery cardiopulmonary indices of CPET for all-cause mortality in CHF patients. **Objective:** The objective of this study is to evaluate the CPET-based recovery cardiopulmonary indices as predictive prognostic factors for death and readmissions. **Methods:** 104 patients with CHF, 81 males (77.9 %), mean age 50.3 years (SD = 13.9), were studied. 39 patients (37.5 %) had diabetes mellitus (DM), 26 (25.0 %) had COPD, 46 (44.2 %) had ischemic heart disease (IHD), and 74 (71.2 %) had hypertension. All patients underwent CPET, including recovery indices, and were followed between 2000 and 2005 for all-cause mortality and for admission (as end point events). Exercise indices were: HR, BP, O₂ consumption (VO₂), O₂ pulse (O₂-P), ventilation (V_e), respiratory exchange ratio (RER), and ventilatory anaerobic threshold (VAT), among others. Recovery indices were: 1/2 time recovery of VO₂ (1/2VO₂-t-Rec.), in sec; 1/2 time recovery of O₂-P (1/2O₂-P-t-Rec.), in sec; and full-time recovery of VO₂ (VO₂-T-Rec.), in minutes (min). **Results:** There were no factors which predicted readmissions. All-cause mortality rate was 24.0 % (n = 25). Significant correlations were demonstrated between all-cause mortality and: peak-V_e (p < 0.007); peak-VO₂ (% pred.; p < 0.001); peak-O₂-P (p < 0.000); VAT (p < 0.020); 1/2VO₂-t-Rec (p < 0.025); 1/2O₂-P-t-Rec (p < 0.019); 1/2VO₂-t-Rec/RER-peak (RER at peak exercise) (p < 0.002); 1/2O₂-P-t-Rec/RER-peak (p < 0.020); 1/2VO₂-t-Rec × RER-peak (p < 0.000); 1/2O₂-P-t-Rec × RER-peak (p < 0.021); 1/2VO₂-t-Rec/RER-max (maximal RER at exercise or recovery) (p < 0.000); 1/2O₂-P-t-Rec/RER-max (p < 0.013); 1/2VO₂-t-Rec × RER-max (p < 0.003), and 1/2O₂-P-t-Rec × RER-max (p < 0.044). By multivariate logistic regression analysis, peak-V_e (regression coefficient = -0.083), 1/2VO₂-t-Rec/RER-peak (regression coefficient = -0.216), 1/2VO₂-t-Rec/RER-max (regression coefficient = 0.272), and DM (regression coefficient = 0.705) were found as the independent predictors of all-cause mortality. **Conclusions:** Recovery cardiopulmonary parameters, as defined in the present study, were found to be significant prognostic predictors at least as exercise indices. Thus, it is suggested to complete the CPET with continuing monitoring during the whole recovery period for further evaluation of CHF patients. **J Clin Basic Cardiol 2012; 15 (online): 13–6.**

Key words: heart failure, cardiopulmonary exercise testing, VO₂ recovery kinetics, prognostic factors

Cardiopulmonary exercise testing (CPET), when properly performed, provides an objective measurement of peak functional capacity and as such has become an important clinical tool for disease severity estimation and for outcome prediction in patients (pts) with chronic heart failure (CHF) [1]. This very test is considered to be the gold standard for the prognostic evaluation of such pts, and low peak-VO₂ is widely recognized as a predictor of poor prognosis [2–4]. Other prognostic factors which have gained interest in recent years were VE/VCO₂ slope and heart rate recovery (HRR) for their prognostic power independent from peak-VO₂ [5–7]. The VE/VCO₂ slope was also found not to be affected by the exercise mode at least in pts with mild CHF [8]. Other studies have shown that oxygen uptake kinetics during recovery from exercise are delayed at more advanced stages of heart failure and that the kinetics of recovery oxygen uptake can be used to measure the functional capacity of CHF pts [9–14]. There is a lack of data regarding the prognostic significance of recovery cardiopulmonary indices in pts with CHF. In the present study, we try to define such a significance of various recovery parameters, taking in account the fact that many pts with CHF do not reach maximal predicted physiological effort during exercise.

Material and Methods

104 pts with CHF, 81 males (77.9 %), mean age 50.3 years (SD = 13.9), were included in the study. They were selected from a pool of 150 pts with CHF who underwent CPET according to the following criteria:

- Age 18–80
- Systolic CHF, with LVEF ≤ 35 %
- Satisfying data inquired for the study

39 patients (37.5 %) had diabetes mellitus (DM), 26 (25.0 %) had COPD, 46 (44.2 %) had ischemic heart disease (IHD), and 74 (71.2 %) had hypertension. All pts underwent CPET, including recovery indices, and were followed from 2000–2005 for all-cause mortality and for admission (as end point events).

Cardiopulmonary Exercise Test

An upright symptom-limited test was performed on an electronically braked cycle ergometer (Ergo-line 800). Exercise was initiated after a 3-minute rest and 2 minutes of free pedalling at a rate of 60 rpm. The effort was then progressively increased by 10–20 Watt/min until the predefined end-point was reached, namely, symptoms, volitional fatigue, or attainment of the target HR. Cardiopulmonary data were collected by an online metabolic unit (CPX Medical Graphics, MN, USA).

Patients breathed through a low-resistance, 2-way valve (Hans-Rudolph, MO, USA) connected to the expiratory limb. The breath-by-breath signals were integrated by a computer to yield 30-sec and averages of HR, minute ventilation, VO₂, VCO₂ and O₂-P (VO₂/HR). VAT was defined as the point at which the ventilatory equivalent of O₂ (V_e/VO₂) increased in the absence of a similar increase in the ventilatory equivalent of CO₂ (V_e/VCO₂), as described by Beaver et al [15]. Blood pressure was measured at rest, every 2 minutes, at peak exercise and during recovery.

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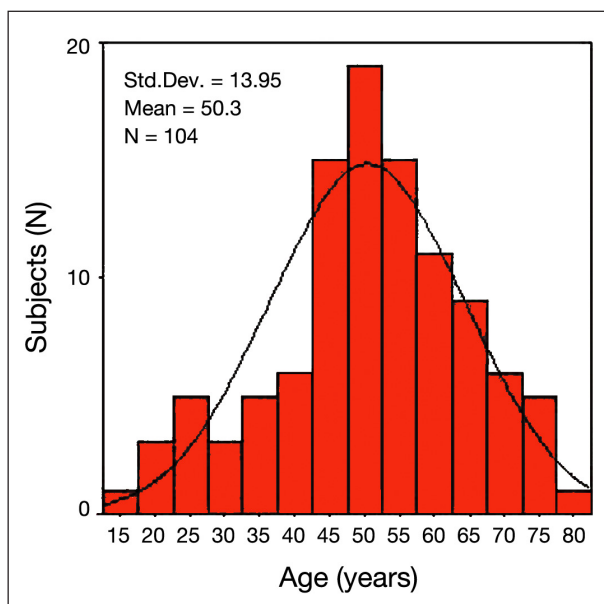


Figure 1. Age variability of the 104 patients participating in the study.

Blood pressure at rest, during exercise, and recovery was measured as for a standard.

Recovery indices were defined as: $\frac{1}{2}$ time recovery of VO_2 ($\frac{1}{2}\text{VO}_2\text{-t-Rec.}$), in sec; $\frac{1}{2}$ time recovery of $\text{O}_2\text{-P}$ ($\frac{1}{2}\text{O}_2\text{-P-t-Rec.}$), in sec; and full-time recovery of VO_2 ($\text{VO}_2\text{-T-Rec.}$), in min, until RER reached value of 1 or less.

Since the pts included in the study had various degrees of CHF, not all of them reached the expected peak exercise, namely, RER of at least 1.15. Therefore, we had to define new combined parameters, involving the RER index, which might minimize the effect of this limitation. These 12 parameters were defined as follows:

1. $\text{VO}_2\text{-T-Rec.}/\text{RER-peak}$ (parameter A)
2. $\frac{1}{2}\text{VO}_2\text{-t-Rec.}/\text{RER-peak}$ (parameter B)
3. $\frac{1}{2}\text{O}_2\text{-P-t-Rec.}/\text{RER-peak}$ (parameter C)
4. $\text{VO}_2\text{-T-Rec.} \times \text{RER-peak}$ (parameter D)
5. $\frac{1}{2}\text{VO}_2\text{-t-Rec.} \times \text{RER-peak}$ (parameter E)
6. $\frac{1}{2}\text{O}_2\text{-P-t-Rec.} \times \text{RER-peak}$ (parameter F)
7. $\text{VO}_2\text{-T-Rec.}/\text{RER-max.}$ (parameter G)
8. $\frac{1}{2}\text{VO}_2\text{-t-Rec.}/\text{RER-max.}$ (parameter H)
9. $\frac{1}{2}\text{O}_2\text{-P-t-Rec.}/\text{RER-max.}$ (parameter I)
10. $\text{VO}_2\text{-T-Rec.} \times \text{RER-max.}$ (parameter J)
11. $\frac{1}{2}\text{VO}_2\text{-t-Rec.} \times \text{RER-max.}$ (parameter K)
12. $\frac{1}{2}\text{O}_2\text{-P-t-Rec.} \times \text{RER-max.}$ (parameter L)

$\text{VO}_2\text{-T-Rec.}$: full-time recovery of oxygen consumption (in minutes); $\frac{1}{2}\text{VO}_2\text{-t-Rec.}$: half-time recovery of oxygen consumption (in seconds); $\frac{1}{2}\text{O}_2\text{-P-t-Rec.}$: half-time recovery of oxygen pulse (in seconds); RER-max.: maximal respiratory exchange ratio achieved in exercise or recovery; RER-peak: peak respiratory exchange ratio at exercise.

Follow-Up Period

All pts were followed from 2000–2005 for at least 2 years per patient, continuously or until death. Follow-up visits of the pts were at the hospital outpatient CHF clinic and/or by their general physician. Each significant event like hospitalization and all-cause mortality were taken from the patients' computerized files for statistical prognostic evaluation.

Statistical Analysis

T-test or Chi-square test were used to compare the parameters between the groups "deceased" vs "survived" and "hospita-

Table 1. Relationship between CPET indices and death (n = 104).

Parameter	Survivors (n = 79)		Deceased (n = 25)		p
	Mean	1 SD	Mean	1 SD	
Age	45.9	12.8	48.7	17.1	0.560
Male %	84.0	–	75.9	–	0.398
Female %	16.0	–	24.1	–	0.398
Peak-Ve*	45.1	13.6	38.7	8.3	0.007
Peak- VO_2 , ml/kg/min	13.1	4.4	11.5	4.2	0.125
Peak- VO_2 , %*	50.2	12.4	40.4	11.1	0.001
Peak- O_2 -Pulse, %*	71.4	19.9	57.1	13.1	0.001
Peak- O_2 -Pulse, ml/beat*	9.0	2.4	6.7	1.7	0.000
VAT, %*	32.6	8.7	27.6	7.2	0.020
peak-HR*	113.9	32.5	101.2	28.1	0.087
WATTS*	56.7	17.4	47.0	18.2	0.025
RER	1.13	0.1	1.14	0.1	0.694
METS*	50.2	12.3	40.0	11.3	0.001
$\text{VO}_2\text{-T-Rec.}$, min	6.9	2.3	6.9	2.4	0.920
$\frac{1}{2}\text{VO}_2\text{-t-Rec.}$, sec*	120	40.7	156	60.4	0.025
$\text{O}_2\text{-Pulse-t-Rec.}$, sec*	206	70.7	250.4	89	0.019
A	6	1.7	5.9	1.9	0.819
B*	107.2	45.4	142.4	50	0.002
C*	183	64	220	75	0.020
D	8	3	7.9	3	0.957
E*	137	56.2	190.2	78.9	0.000
F*	231.6	99	288	100.3	0.021
RER-max	1.4	0.2	1.4	0.2	0.831
G	4.8	1.3	4.9	1.6	0.842
H*	84.3	37.4	119	47.1	0.000
I*	147.6	50.5	182.4	63.3	0.013
J	10.2	4.3	9.8	4	0.696
K*	172	70	228.4	80	0.003
L*	296.3	120	355.7	130	0.044
HRR	22.5	11.6	23.8	12.6	0.064

Ve: ventilation in l/min; VO_2 : oxygen consumption; $\text{O}_2\text{-Pulse}$: oxygen pulse; VAT: ventilatory anaerobic threshold; HR: heart rate; RER: respiratory exchange ratio; METS: metabolic units; T,t: time; rec: recovery; A–L parameters: see Methods section; HRR: heart rate recovery

talized" vs "not hospitalized". Each parameter was presented as mean $\pm$ 1 SD. Multivariate logistic regression analysis models were used for identification of non-dependent prognostic parameters. $P < 0.05$ was considered statistically significant.

Results

Figure 1 shows the patients' age spread.

The all-cause mortality rate was 24.0 % (n = 25). Significant correlations were demonstrated between all-cause mortality and peak-Ve ($p < 0.007$); peak- VO_2 (% pred.; $p < 0.001$); peak $\text{O}_2\text{-P}$ ($p < 0.000$); VAT ($p < 0.020$); $\frac{1}{2}\text{VO}_2\text{-t-Rec}$ ($p < 0.025$); $\frac{1}{2}\text{O}_2\text{-P-t-Rec}$ ($p < 0.019$); $\frac{1}{2}\text{VO}_2\text{-Rec}/\text{RER-peak}$ (RER at peak exercise) ($p < 0.002$); $\text{VO}_2\text{-T-Rec}/\text{RER-peak}$ ($p < 0.020$); $\frac{1}{2}\text{VO}_2\text{-t-Rec} \times \text{RER-peak}$ ($p < .0000$); $\text{VO}_2\text{-T-Rec} \times \text{RER-peak}$ ($p < 0.021$); $\frac{1}{2}\text{VO}_2\text{-t-Rec}/\text{RER-max}$ (maximal RER at exercise or recovery) ($p < 0.000$); $\text{VO}_2\text{-T-Rec}/\text{RER-max}$ ($p < 0.013$); $\frac{1}{2}\text{VO}_2\text{-t-Rec} \times \text{RER-max}$ ($p < 0.003$); $\text{VO}_2\text{-T-Rec} \times \text{RER-max}$ ($p < 0.044$; Table 1).

Table 2. Relationship between CPET indices and hospitalizations (n = 104).

Parameter	Hospitalizations (n = 97)	1 SD	Not hospitalized (n = 7)	1 SD	p
Age	50.7	14.0	49.5	13.8	0.669
Male, %	78.6		45.5		0.809
Female, %	21.4		54.5		0.809
Peak-Ve	42.5	13.2	71.3	11.9	0.276
Peak-VO ₂ , %	46.6	13.7	32.8	10.5	0.152
Peak-VO ₂ , ml/kg/min	12.7	4.8	12.7	4.2	0.968
Peak-O ₂ -Pulse, %	66.5	19.9	119.0	18.3	0.244
Peak-O ₂ -Pulse, ml/beat	8.7	2.6	8.5	2.4	0.647
VAT _i , %	30.6	9.0	55.9	7.6	0.290
Peak-HR	106.9	28.0	1.1	37.7	0.069
WATTS	53.9	19.8	51.0	13.9	0.603
Peak-RER	1.1	0.1	1.1	0.1	0.242
METS	46.1	13.5	51.0	10.6	0.071
VO ₂ -T-Rec, min	7.14	1.7	6.84	2.6	0.481
½VO ₂ -Rec, sec	126.8	55.7	129.3	56.4	0.831
½O ₂ -P-t-Rec, sec	217.8	75.6	216.4	73.5	0.938
A	6.2	1.2	6	1	0.587
B	11.9	52	116.6	46.6	0.650
C	192.5	65	191.9	66	0.965
D	8.3	2.4	7.9	3.4	0.504
E	141	62	151.6	67	0.449
F	241	101	246	106.4	0.825
RER-max	1.45	0.2	1.42	0.2	0.437
G	5	1.1	4.8	1.5	0.596
H	85.9	42.3	95	42.1	0.314
I	153.6	56.5	156.6	60.2	0.802
J	10.5	3.2	4.6	9.9	0.549
K	185	82.6	184	88	0.976
L	314.4	115.5	308.5	115.3	0.840
HRR	21.3	10.8	25.9	13.3	0.065

Ve: ventilation in l/min; VO₂: oxygen consumption; O₂-Pulse: oxygen pulse; VAT: ventilatory anaerobic threshold; HR: heart rate; RER: respiratory exchange ratio; METS: metabolic units; T,t: time; Rec: recovery; A-L parameters: see Methods section; HRR: heart rate recovery

The main results are summarized in Tables 1–4.

There were no factors which predicted hospitalizations. The main reason for this finding might be the fact that only 7 out of the 104 pts were not hospitalized during the follow-up period (Table 2).

By multivariate logistic regression analysis, peak-Ve (regression coefficient = -0.083; p = 0.043), and ½VO₂-t-Rec/RER-max (regression coefficient = 0.272; p = 0.047) were found as independent predictors of all-cause mortality. ½VO₂-t-Rec/RER-peak (regression coefficient = -0.216; p = 0.068) and DM (regression coefficient = 0.705; p = 0.062) were borderline independent predictors (Table 3).

Table 4 shows that background diseases of IHD or DM are prognostically significant for death while COPD or HTN are not.

Discussion

In daily practice, peak-VO₂ significantly influences decisions regarding the management of pts with CHF and is consid-

Table 3. Independent relationship between CPET and disease parameters and death.

Parameter	Regression factor	Standard error	p
Peak-Ve	-0.083	0.041	0.043
O ₂ -Pulse	-0.025	0.020	0.212
½VO ₂ -t-Rec.	-0.105	0.098	0.283
O ₂ -Pulse-t-Rec.	0.004	0.215	0.983
B	-0.216	0.118	0.068
C	0.141	0.127	0.269
E	0.010	0.032	0.767
F	0.038	0.084	0.650
H	0.272	0.137	0.047
I	-0.157	0.118	0.184
K	0.067	0.046	0.139
L	-0.041	0.049	0.408
IHD	0.716	0.700	0.307
Male gender	1.265	0.946	0.181
DM	0.705	0.743	0.062

Ve: ventilation in l/min; VO₂: oxygen consumption; O₂-Pulse: oxygen pulse; T,t: time; Rec: recovery; A-L parameters: see Methods section; IHD: ischemic heart disease; DM: diabetes mellitus

Table 4. Relationship between background diseases and death (n = 104).

Parameter	Survivors	Deceased	p
DM*	31.6	56.0	0.028
COPD	24.1	28.0	0.691
IHD*	38.0	64.0	0.022
HTN	73.4	64.0	0.365

IHD: ischemic heart disease; DM: diabetes mellitus; HTN: hypertension; COPD: chronic obstructive pulmonary disease

ered to be an important mortality predictor in such pts [15–21]. However, this very index depends on the mode of exercise particularly in such CHF pts [8, 22–24]. The independent prediction power of other indices, like Ve/VCO₂ or heart rate recovery (HRR), are still to be proven. On the other hand, exercise intolerance in CHF pts is hard to quantify since the end point for the test is usually subjective, depending on motivation of the pt or the examiner. Even the VAT, which is independent of the pt motivation, might be limited for prognostic evaluation especially in those with undetectable VAT [25, 26]. Therefore, it is necessary to identify new prognostic parameters which may strengthen the ability for such assessment of CHF pts. The present study demonstrates several recovery, as well as exercise, cardiopulmonary parameters as prognostic predictors in pts with CHF. All-cause mortality is significantly correlated with several exercise CPET indices but not less significantly with recovery indices as well (Table 1). According to regression analysis, Peak-Ve, ½VO₂-t-Rec/RER-peak and ½VO₂-t-Rec/RER-max are the most significant independent predictors of prognosis in CHF.

Exercise Indices

The study by Mancini et al [3] is still considered a cornerstone of the prognostic power of peak-VO₂ in the evaluation of CHF pts. Nevertheless, it is important to prove that

exercise testing was truly maximal, a task which is quite hard to achieve in many CHF pts. In our study it was shown that this very parameter, especially when expressed by the percentage of maximal predicted values, has quite a strong predictive power for death. The O_2 -pulse, which reflects the SV, has even more significant predictive power in such an evaluation, as can be seen in Table 1. The prognostic significance of the O_2 -Pulse is barely discussed in the medical literature.

VAT is considered as a submaximal index of exercise capacity, independent of patient motivation. A failure to reach VAT suggests poor motivation or non-cardiac limitation of exercise tolerance. Such a failure may be observed in a large proportion of CHF pts [25].

VAT in the present study has been shown as a strong predictor of death in CHF pts (Table 1).

The only exercise index that was found to be significantly independent for all-cause mortality was peak- V_e (Table 3).

It is a lack of information about this factor by itself but its relationship to VCO_2 is demonstrated to be an important prognostic predictor, as V_e/VCO_2 slope > 34 was associated with worse prognosis in CHF [27].

CHF pts with severe exercise intolerance need more objective indices for evaluation of maximal effort since their motivation to reach such an effort is limited or lacking [28].

Recovery Indices

In our study, we tried to evaluate the contribution of recovery cardiopulmonary indices in the prognostic prediction of CHF pts. To add objective strength, we defined additional parameters in which the already defined recovery indices were multiplied by or divided in the peak-RER achieved during exercise or maximal RER which was achieved during the whole recording within exercise or recovery period as well. Since such RER values might be affected by subjective factors like patient motivation, where pts do not reach the peak, physiological expected, RER of ≥ 1.15 , as shown in the Mezzani et al study [29], these new parameters might suggest a physiological correction to the bare recovery indices, which might be subjective as well.

Table 1 demonstrates that the recovery indices $\frac{1}{2}VO_2$ -t-recovery and $\frac{1}{2}O_2$ -P-t-recovery are significant death-predicting factors in CHF, strengthened by the high significance of these very factors multiplied by or divided in the RER values (parameters: B, C, E, F, H, I, K, and L in Table 1). Nevertheless, from all these parameters, only factor H ($\frac{1}{2}VO_2$ -t-recovery/RER-max) was found to be a significantly independent one for death prediction, while factor B ($\frac{1}{2}VO_2$ -t-recovery/RER-peak) was borderline, as well as DM (Table 3).

Limitations and Conclusions

The main limitations of the present study were its retrospective characteristics and the relatively small number of patients. Such a sample does not allow to calculate quantitatively the new recovery parameters checked in this study in relationship to prognostic significance. Nevertheless, these findings suggest that recovery indices should be recorded following the CPET for further prognostic evaluation in CHF patients. More studies, and with larger patient samples, are necessary for further quantitative assessment of recovery parameter values and their contribution to evaluate prognosis in CHF patients.

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