



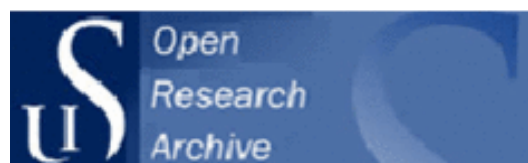
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***Plasma TNF correlates with mRNA expression of TNF and mitochondrial transcription factors in skeletal muscle in patients with chronic heart failure treated with cardiac resynchronization therapy
- potential role in myopathy -***

Research letter R2

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Chronic heart failure (CHF) is characterized by inflammation, and skeletal muscle myopathy including impaired fiber type distribution and reduced capillary density, reduced cytochrome oxidase activity and reduced mitochondrial density. The myopathy is associated with activation of the IL-6-CRP pathway and the prototypical inflammatory cytokine tumor necrosis factor (TNF) with alterations in the mRNA expression of enzymes essential in mitochondrial biogenesis. Central in this process are Peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α), nicotinamide phosphoribosyltransferase (NAMPT), nicotinamide adenine dinucleotide (NAD⁺) and mitochondrial transcription factor (TFAM).

The covariance over time, between plasma levels of TNF and skeletal muscle mRNA expression of this pro inflammatory cytokine, and the correlation between TNF, and mRNA expression of enzymes essential in mitochondrial biogenesis and skeletal muscle pathology is previously not evaluated in patients with CHF on stable medical treatment.



The methods have been described previously¹ and are briefly presented here.

Patients. Fourteen out of 21 patients with stable CHF and left bundle branch block (LBBB) who were candidates for CRT, prospectively recruited from the outpatient HF clinic at the Stavanger University Hospital, had acceptable skeletal muscle biopsies suitable for mRNA analysis (Table 1). All tests were performed at baseline before insertion of a cardiac resynchronization treatment (CRT) device and after 6 months of CRT. There was no change of medication during this period.

CRT insertion. A cardiac resynchronization device was inserted with conventional technique.

Functional capacity. The patients were evaluated on an upright, electrically braked ergometer bicycle (Model KEM III, Mijnhardt, S.V. Bunnik, The Netherlands) using a 15 Watt /min ramp protocol. Gas exchange data were collected continuously with an automated breath-by system 2001, (Medical Graphics Corporation, St. Paul, MN).

Blood sampling protocol. After resting for 30 minutes, blood was drawn into pyrogen-free blood collection tubes with EDTA as anticoagulant. Plasma tubes were immediately immersed in melting ice, and centrifuged at 2500g for 10 minutes at 4°C within 20 minutes. All samples were stored at -80°C.

Plasma analysis. Plasma levels of TNF were measured using a multiplex cytokine immunoassay (Bio-Plex Human Cytokine Plex Panel, Bio-Rad Laboratories, Hercules, CA) on a Multiplex Analyser (Bio-Rad Laboratories).

Muscle biopsies. Open muscle biopsies were harvested with surgical technique before implantation of the CRT device and after 6 months of CRT as previously described². The second biopsy was harvested from the contra lateral quadriceps muscle.

Electron microscopy. Muscle biopsies were immediately fixed for minimum 24 hours in McDowell's fixative. Post-fixation was performed in 1% aqueous OsO₄. The specimens were dehydrated in series of graded ethanol and thereafter embedded in Epoxy resin. Semi-thin sections (2 µm) of 4 blocks from each biopsy were stained with 1% Toluidine blue. Ultra-thin sections (70 nm) from the selected blocks were placed on single hole copper grids covered with "formvar" film. The ultra thin sections were contrasted with 5% uranyl acetate and subsequently with Reynolds lead citrate. The sections were examined in a JEOL 1010 electron microscope.

Stereology Point-counting stereology on the electron micrographs was used for morphometric registration. The electron micrographs were covered by a 1x1 cm square lattice. Grid points lying in mitochondria were counted. For each section, the number of counted grid points from 10 micrographs were summarized and expressed as absolute values.

Real-time quantitative RT-PCR. Total RNA was extracted from skeletal muscle using TRIzol (Invitrogen, San Diego, CA), DNase treated, cleaned up using RNeasy Mini Columns (Qiagen, Hilden, Germany), and stored at -80°C. cDNA was synthesized using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantification of gene expression was performed using the ABI Prism 7500 (Applied Biosystems), Power SYBR Green Master Mix (Applied Biosystems), and sequence-specific PCR primers designed using the Primer Express software, version 3.0 (Applied Biosystems).

Ethics The study was approved by the regional ethics committee and conducted according to the Declaration of Helsinki. Informed consent for participation was received from all individuals.

Statistics All data were analysed using SPSS 22.0 (IBM Corp., Armonk, NY). All differences and ratios were analysed for normality using the Kolmogorov Smirnov test with Lilliefors significance correction, Shapiro Wilk tests and normal plots. Non-parametric tests (Wilcoxon sign rank tests) were applied to test for changes over time if the data were not normally distributed. Otherwise t-tests were used. Data for changes in gene expression are expressed as ratios calculated by dividing the value at 6 months with the value at baseline (6 month value/baseline value). Other changes are reported as differences. Pearson's correlation tests were employed if the data were normally distributed and Spearman's rho correlation test was employed if this was not the case. The value of significance was set to $p < 0.05$. All muscle and plasma analysis were performed blinded for patient identity and sequence.



As reported before, CRT during 6 months was associated with improved functional capacity, improvement in VE/VCO₂ slope, increased capillary density in skeletal muscle and reduced left ventricular end diastolic diameter. There was a trend towards a decrease in plasma levels of TNF (pg/ml) (48.3 ± 30.2 vs 41.3 ± 26.0 , $p = \text{NS}$), and a trend towards an increase in mitochondrial density (186 ± 73 vs 205 ± 74 , $p = \text{NS}$)¹. After careful quality analysis, RNA of sufficient quality was available

for gene expression analysis in 14 of the patients. One of the TNF samples was excluded because of being extreme outlier. Additionally 2 samples were excluded due to reduced quality and shortage of sample volume. There were no statistical significant changes in mRNA expression of TNF or enzymes essential in mitochondrial biogenesis. However, there was a statistical significant correlation between changes in plasma levels of TNF and changes (ratio) in muscular expression of mRNA levels of TNF (N=14, Figure 1A; $R=0.56$, $p<0.05$). There was also a highly statistical significant correlation between changes in plasma levels of TNF and changes (ratio) in muscular expression of mRNA levels of TFAM (Figure 1B; $R=-0.81$, $p<0.001$) and NAMPT (Figure 1C; $R=-0.81$, $p<0.001$).

In addition there was a statistical significant correlation between changes in skeletal muscle mRNA levels of TNF and changes in mitochondrial density (Figure 1D $R=-0.561$, $p=0.037$). Moreover changes in plasma TNF were correlated with changes in number of intramuscular lipid droplets ($R=0.52$, $p=0.02$). “There was also a trend towards a statistically significant correlation between alterations in plasma levels of TNF and changes in fibre diameter.

Moreover, alterations in plasma Interleukin-6 correlated with fibre diameter ($r=-0.58$, $p=0.009$)



(i) TNF is produced by immune-competent cells such as macrophages and lymphocytes, but is also released from a number of other cells including cells within the myocardium³ and skeletal muscle⁴. In the current study we found that changes in *intramuscular* gene expression of TNF correlated with changes in plasma TNF suggesting that skeletal muscle TNF expression might be a factor for increased levels of plasma TNF in CHF patients.

(ii) The changes in plasma levels of TNF were negatively correlated with changes in mRNA expression of key regulators of skeletal muscle mitochondrial biosynthesis (NAMPT and TFAM) suggesting that skeletal muscle wasting in CHF is partially mediated via activation of the prototypical inflammatory cytokine TNF. Indeed, chronic exposure to TNF has been shown to promote muscle weakness⁵. Activation of TFAM has been shown to be central in the regulation of mitochondrial biogenesis in regenerating skeletal muscle⁶. This activation is also seen in exercise training, which induces activation of PGC-1 α ⁷. In addition exercise-induced NAMPT-activity, which indirectly activates PGC-1 α , has been shown to correlate with mitochondrial cellular content⁸.

(iii) Supporting the negative correlation between TNF and TFAM and NAMPT, we also found a statistically significant correlation between changes in intramuscular mRNA levels of TNF and changes in skeletal muscle mitochondrial density as well as parameters of skeletal muscle structure (i.e., lipid droplets). These findings in clinical CHF are in line with the findings in an experimental model of pacing-induced cardiac failure where mitochondrial dysfunction was associated with increased levels of TNF⁹. The chronically reduced skeletal muscle perfusion in inactive patients with

CHF results in hypoxia-induced production of reactive oxygen species, which in turn may enhance TNF expression.¹⁰ This pathogenic loop could contribute to skeletal muscle pathology in CHF, which again may contribute to the progression of CHF with TNF as a missing link. **This is further supported by the findings of reversal of muscle abnormalities after exercise training programmes¹¹.** However, correlations in more general terms do not necessarily mean any causal relationship. On the other hand, the findings are in accordance with recent research, which indicates that levels of biomarkers that reflect pro-inflammatory and pro-fibrotic processes are associated with differential effect of exercise on functional capacity in patients with heart failure¹².



Plasma levels of TNF **are closely related** with skeletal muscle mitochondrial biosynthetic enzymes and moderately with skeletal muscle gene expression of TNF, which in turn co vary moderately with skeletal muscle mitochondrial density. **The lack of significant variation in TNF levels over time, the small sample size and missing data are limitations of the study.**

Our findings may suggest that TNF could contribute to skeletal muscle myopathy via down regulation of mitochondrial biosynthesizing enzymes and thus reduced mitochondrial density.

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Authorship: AIL, PA and AY contributed to the conception or design of the work. AIL, CO, PSM, TV, AY, BH, PA, JTK and SL contributed to the acquisition, analysis, or interpretation of data for the work. AIL, AY, PA and PSM drafted the manuscript. All critically revised the manuscript. All gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

Figure legend

Figure 1.

Correlations between changes in plasma levels and skeletal muscle gene expression of tumor necrosis factor (TNF), (A), correlations between changes in plasma levels of TNF and changes in gene expression of *mitochondrial transcription factor A* (TFAM) (B), correlation between changes in skeletal muscle gene expression of TNF and mitochondrial density (C), correlations between changes in plasma levels of TNF and *nicotinamide phosphoribosyltransferase* (NAMPT) (D).

Figure

Figure 1. Correlations between changes in plasma TNF and skeletal muscle mRNA expression of TNF (A), plasma TNF and TFAM (B), plasma TNF and NAMPT (C) and mRNA TNF and mitochondrial density (D).

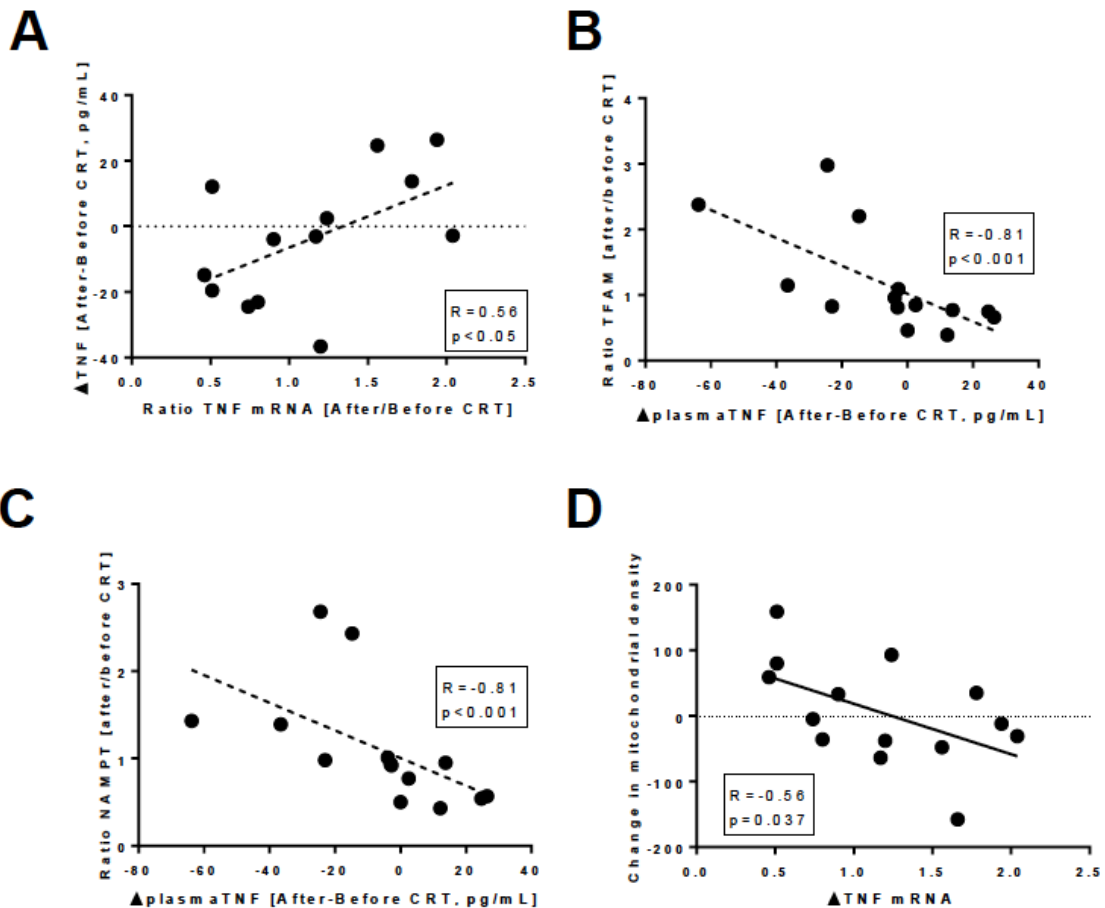


Table 1. Baseline characteristics.

	Heart failure patients (n=14)
Male sex, n (%)	10 (70%)
Age, years	72 [63.5, 76.3]
Etiology (IHD/DCM), n	9/5
NYHA: II/III, n	3/11
LV ejection fraction, %	23.5 [20.0, 30.0]
LVEDD, cm	6.8 [6.2, 7.2]
BMI, kg/m ²	27.5 [25.1, 29.2]
Peak VO ₂ , mL/kg/min	13.3 [11.7, 15.5]
Medication (%)	
ACE inhibitor/ARB	100
β-blocker	100
Diuretics	93
Aldosterone antagonist	43

Values are presented as number (%) or median [interquartile range]. IHD, ischaemic heart disease; DCM, dilated cardiomyopathy; NYHA, New York Heart Association; LVEDD, left ventricle end-diastolic dimension; BMI, body mass index; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

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