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# Oxidative stress implication in a new phenotype of amyotrophic quadricipital syndrome with cardiac involvement due to lamin A/C mutation

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**Abstract**

This study aimed at evaluating OS in an amyotrophic quadricipital syndrome with cardiac impairment in a family of 80 members with a mutation in lamin A/C gene. Twelve patients had cardiac involvement (5 cardiac and skeletal muscles impairment). OS was evaluated in blood samples (thiobarbituric acid-reactive substances (TBARS), carbonylated proteins (PCO)) 6 "affected patients" with phenotypic and genotypic abnormalities without heart failure and 3 "healthy carrier" patients. OS was higher in affected patients than in healthy, as shown by the higher TBARS and PCO values. Patients with cardiac and peripheral myopathy exhibited a higher OS than patients with only cardiac disease (TBARS: $1.73 \pm 0.05$ vs. $1.51 \pm 0.04$ mmol/l ($p = 0.051$), PCO: $2.73 \pm 0.34$ vs. $0.90 \pm 0.10$ nmol/mg protein ($p = 0.47$)), and with healthy carriers patients (TBARS: $1.73 \pm 0.05$ vs. $1.16 \pm 0.14$ mmol/l ($p = 0.05$), PCO: $2.73 \pm 0.34$ vs. $0.90 \pm 0.20$ nmol/mg protein ($p = 0.47$)).

OS may thus contribute to the degenerative process of this laminopathy. ROS production occurs, prior to heart failure symptoms. We suggest that the extent activation may also promote the variable phenotypic expression of the disease.

**Keywords:** *Cardiomyopathy, oxidative stress, heart failure, TBARS*

## Introduction

A growing body of evidence resulting from both clinical and experimental data has emerged over the past years suggesting a role of reactive oxygen species (ROS) in the pathophysiology of some neurological diseases [1–4].

Deposition of amyloid-β (Aβ) is thought to play a central role in the development of Alzeihmer's disease. This increase in Aβ production is followed by chelation of transition metal ions by Aβ, accumulation of Aβ metal lipoprotein aggregates, production of ROS and neurotoxicity [1]. In Parkinson's disease, a high level of lipid peroxidation, an increase in iron level and a disturbance in the mitochondrial respiratory chain have been reported, suggesting an increased production of toxic free radicals [2–4].

In cardiac disease, oxidative stress (OS) is increased in the ischemia/reperfusion injury, in which reperfusion has been reported as a potent stimulus for ROS production. Accordingly, myocardial stunning is also considered as a manifestation of reperfusion injury [5]. Recent data also support a contributory role of ROS in the pathophysiology of cardiac hypertrophy

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[5]. Treatment with the free radical scavenger N-2-mercaptopropionyl glycine reduces cardiac hypertrophy [6]. Increased levels of plasma OS markers have been found in patients with congestive heart failure particularly in those with ischaemic heart disease [7]. Finally, ROS have also been reported to trigger apoptosis of cardiac myocytes [8], but this source and target remain to be clarified [9]. Therefore, all studies had evaluated symptomatic patients (heart failure, ischemic disease).

The role of OS in human muscle diseases is less known. OS is rarely studied in pathologies associating cardiac and muscular involvement like Emery–Dreyfuss syndrome, muscular dystrophy, limb-girdle muscular dystrophy, although it has been reported to play a role in the pathogenic cascade of hereditary inclusion body myopathies [10].

In this study we hypothesized a possible role of OS in patients symptoms with a new phenotype of myopathy with cardiac involvement associated with a lamin A/C mutation without heart failure [11,12].

## Patients and methods

### *Study subjects*

In the family studied, cardiac involvement occurred at 37.1 ± 7.3 years. It was characterized by abnormalities of cardiac conduction (atrioventricular block) and arrhythmias (atrial fibrillation, ventricular ectopic beats and ventricular tachycardia) and by a progressive dilated cardiomyopathy [11]. Two patients had NYHA III–IV class of heart failure. Muscular disease occured after the age of 40 years, consisting of bilateral quadriceps myopathy. By contrast with previously reported cases [13,14], all patients with neurological symptoms had cardiac abnormalities and cardiac disorders preceded quadriceps myopathy involvement. The pedigree demonstrated that transmission was autosomal dominant. The genetic study identified a missense mutation in the lamin A/C gene (R 377 H) [12].

The study included 9 subjects of an 80-member family and focused on the second generation and 2 patients of the third. We included 3 "healthy carriers" without genetic abnormalities ("control group") and 6 patients ("affected patients") with a known cardiac and neurologic status and genetic abnormalities (Figure 1). All affected patients exhibited were in NYHA I–II class. None was treated with statin, aspirin nor angiotensin converting enzyme inhibitors (ACE). Moreover none had ever cigarette smoking or diabetes. No differences in lifestyle, mobility nor diet were identified. Written informed consent was obtained in accordance with the study protocol approved by the local ethic committee.

### *Biological parameters*

Circulating blood levels of total cholesterol (normal ranges: 4.4–6.1 mmol/l), LDL-cholesterol (2.6–4.1 mmol/l), triglycerides (0.5–1.4 mmol/l), phospholipids (2.50–3.60 mmol/l) were measured using

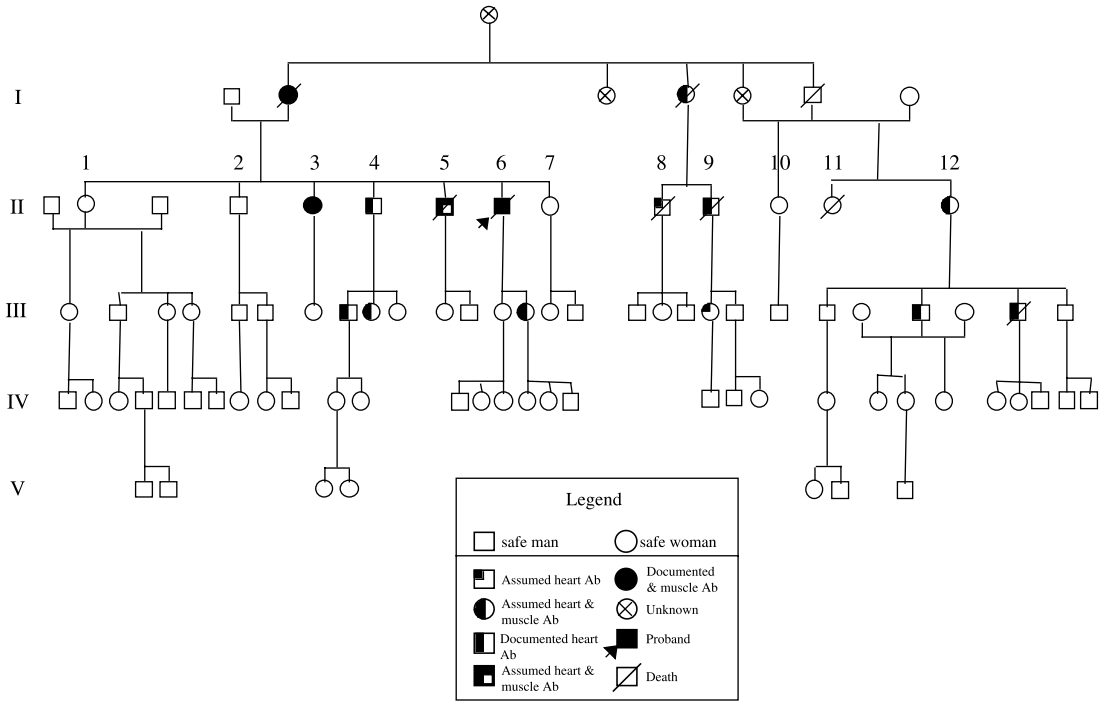


Figure 1. Pedigree of the family.

classical automated enzymatic methods [15–17]. HDL-cholesterol (1.3–1.9 mmol/l) was assayed by a direct method described by Sugiuchi et al. [18], combining action of polyethylene glycol-modified cholesterol esteraseandoxidase with a-cyclodextrin sulfate, using reagents from Randox⋆ (Montpellier, France). Apolipoprotein E (apo E) phenotype was determined by agarose gel electrofocusing performed with a Multiphor System⋆ (Pharmacia) followed by immunoblotting using goat polyclonal antibody to apo E (Interchim⋆) and rabbit IgG anti-goat horseradish peroxidase conjugate (Sigma⋆). Inflammatory status was assessed by CRP (<5 mg/l) measurement. A systematic determination of creatine kinase activity, troponin and myoglobin concentrations was performed.

OS parameters were measured as previously described [19]. Plasma thiobarbituric acid-reactive substances (TBARS) were assayed using a spectrofluorimetric method after condensation with thiobarbituric acid [20]. Plasma and urine 8-epiPGF2α were determined by an immunoenzymatic method (Cayman Chemical Company, ref 516351), as previously described [21]. Carbonylated proteins were assayed in plasma using a spectrophotometric method after reaction with 2,4-dinitrophenylhydrazine [22]. Plasma α-tocopherol, vitamin A and β-carotene were determined by reverse phase HPLC [23]. The total antioxidant status (TAS) was measured in plasma by means of a commercial kit (Randox⋆, Roissy, France) based on the method developed by Miller et al. [24] using 2,2′-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (ABTS). The inducible nitric oxide synthase II (iNOS) activity was determined in a cardiac biopsy specimen from a patient who died suddenly (patient II6), using a method based on the conversion of L-arginine into L-citrulline after homogenisation in a sodium phosphate buffer at pH 7.4 containing 20 mmol/l HEPES, 1 mmol/l EGTA, 1 mmol/l dithiothreitol, 0.32 mol/l sucrose and 10 mg/l leupeptin and pepstatin [25]. A control sample contained aminoguanidine (A 7009, Sigma) as preferential inhibitor of NOS-II activity. Proteins were assayed in the homogenates by the method of Bradford [26] using the Bio-Rad protein Asay kit (500–0006C, Biorad). We have studied the NOS-I immunolocalization with specific antibodies.

*Statistical analysis*

Data are presented as mean ± SEM. Quantitative variables were compared using the Mann–Whitney test, and $p < 0.05$ was considered as significant.

## Results

We studied 9 patients (4 females and 5 males) aged 51.7 ± 10.1 years (range: 41–66 years). Among them, 3 patients were healthy carriers of mutation (II1, II2, II7) and 6 were clinically affected (II3, II4, II6, II11, III24, III25) (Figure 1). The last group has been subdivided into two groups: 3 patients had only cardiac disease (II4, II11, III25) and 3 patients had both cardiac and quadriceps disease (II3, II6, III24).

*Biochemical data*

Baseline characteristics of patients for CRP, total cholesterol, triglycerides, creatine kinase and troponin (Table I) were within normal ranges. Myoglobin values were slightly elevated in comparison with normal value. In the studied family, the absence of any inflammatory syndrome and the normal serum creatine kinase (CK) activity were in accordance with the inclusion criteria for the diagnosis of muscular dystrophy showing a slowly progressive symmetrical proximal weakness, normal to mildly raised serum CK activity, myopathic changes on electromyography and muscle biopsy without inflammation, and normal dystrophin analysis of the muscle tissue [11,27–29]. All patients had an E3/E3 phenotype of apolipoprotein E.

*Oxidative stress data*

Plasma TBARS levels were elevated in affected patients vs. healthy carriers subjects (respectively 1.62 ± 0.12 vs. 1.16 ± 0.14 μmol/l; $p = 0.05$) (Table II). Noteworthy, lipid peroxidation was higher among patients with cardiac and muscle involvement than in the group of patients with only cardiac disease (TBARS 1.73 ± 0.05 vs. 1.51 ± 0.04 μmol/l, $p = 0.051$) (Table III).

Carbonylated protein levels were higher in affected patients than in healthy carriers (1.81 ± 0.94 vs.

Table I. General biological parameters in healthy carriers and affected patients (means ± SD).

| | Usual values | Healthy carriers | Affected patients |
|---|---|---|---|
| CRP (mg/l) | <5 | 1.43 ± 1.39 | 3.47 ± 1.64 |
| Cholesterol (mmol/l) | 4.40–6.10 | 6.15 ± 0.24 | 6.01 ± 0.56 |
| Triglycerides (mmol/l) | 0.50–1.40 | 0.74 ± 0.24 | 1.48 ± 0.90 |
| CK (U/l) | 25–195 | 90 ± 19 | 164 ± 14 |
| Troponin (μg/l) | <0.2 | <0.1 | <0.1 |
| Myoglobin (μg/l) | <50 | 54 ± 6 | 62 ± 9 |

Table II. Oxidative stress status in healthy carriers and affected patients (means ± SD).

| | Usual values | Healthy carriers | Affected patients |
|---|---|---|---|
| TBARS (μmol/l) | 0.60–1.20 | 1.16 ± 0.14 | 1.62 ± 0.12 |
| Plasma 8-epi PGF2α (pg/ml) | 40–100 | 88 ± 17 | 79.5 ± 16 |
| Urine 8-epi PGF2α (ng/mmol creatinine) | 50–100 | 77 ± 7 | 106 ± 26.5 |
| Carbonylated proteins (nmol/mg protein) | 0.55–0.9 | 0.90 ± 0.20 | 1.81 ± 0.94 |
| Vitamin A (μmol/l) | 1.5–2.6 | 2.5 ± 0.5 | 2.7 ± 0.25 |
| β-carotene (μmol/l) | 0.20–0.80 | 0.54 ± 0.19 | 0.32 ± 0.14 |
| α-tocopherol (μmol/l) | 20–37 | 42.2 ± 4.6 | 33.7 ± 7.28 |
| TAS (mmol/l) | 1.30–1.90 | 1.23 ± 0.09 | 1.05 ± 0.05 |

Usual values were obtained in 100 healthy subjects aged 20–45 years without cardiovascular risk factors (diabetes, smoking, hypertension, dyslipidemia). TBARS: thiobarbituric acid-reactive substances; TAS: total antioxidant status.



0.90 ± 0.20 nmol/mg protein; $p = 0.05$). This difference is associated significantly higher values of carbonylated proteins in patients exhibiting both cardiac and quadriceps involvement as compared to those with isolated cardiac disease (respectively, 2.73 ± 0.34 vs. 0.90 ± 0.10 nmol/kg, $p = 0.47$).

TAS was decreased in all patients (1.05 ± 0.06 mmol/l in affected patients vs. 1.23 ± 0.09 mmol/l in healthy patients), thus supporting the presence of an OS in this population. Nevertheless, the liposoluble antioxidants, i.e. β-carotene, α-tocopherol and vitamin A, ranged within normal values: (respectively 0.32 ± 0.14 μmol/l, 33.7 ± 7.28 μmol/l and 2.7 ± 0.25 μmol/l in affected patient, vs. 0.54 ± 0.19 μmol/l, 42.2 ± 4.6 μmol/l and 2.5 ± 0.5 μmol/l in healthy carriers). Isoprostane (8-epi-PGF2α) concentration was also within normal values in plasma and urines.

Determination of cardiac NOS-II activity in the patient who suddenly died gave a mean value of 352 fmol $mg^{-1}$ $min^{-1}$ (values obtained from multiple left ventricle samples between 132 and 784 fmol $mg^{-1}$ $min^{-1}$). This variation in the results is perhaps due to an heterogeneity of the oxidative stress effects in the heart muscle. In addition, NOS-I is expressed in the cardiomyocyte. NOS-I immunoreactivity appeared to be increased at the sarcolemma level in failing hearts, which suggests a partial translocation of NOS-I from the cytosol to the membrane in heart failure (Figure 2).

## Discussion

The present study reports OS status in 9 members of a French family affected with a new phenotype due to lamin A/C mutation without heart failure symptoms. The clinical and genotypic characteristics of the family have previously been reported [11,12]. For the first time, we describe the OS profile observed in these patients and its dependence on the phenotypic expression of the disease (i.e. isolated cardiac involvement or both cardiac and skeletal muscles involvement). Several mechanisms have been hypothesized to explain cardiac failure in laminopathies: (1) an impaired contractile performance of the sarcomere; (2) a reduced transmission of contractile force; (3) any associated gene mutation with unknown mechanisms [11].

This study demonstrates increased plasma TBARS and carbonylated proteins levels among affected patients with laminopathies. A significant increase in carbonylated proteins among patients with cardiac and neurologic involvement is observed, associated with a trend to significant by enhanced TBARS values. Moreover, the TAS was reduced in patients without any abnormalities of the liposoluble antioxidants assayed.

Thus, OS could play an important role in the pathogenesis of cardiac dysfunction. Previous studies evaluated patients with severe heart failure symptoms (NYHA IV class). They showed that lipid peroxidation was directly related to the severity of heart failure. Also, this mechanism was more significantly pronounced in this setting, particularly when compared in patients with ischemic heart disease [29–31]. The mechanisms linking heart failure to OS involve several steps [30]: (1) the sarcoplasmic reticulum (SR) calcium cycling defects could provide a potential molecular mechanism leading to progressive cardiac dysfonction altering the contraction/relaxation coupling; (2) a decreased ATP levels may lead to the observed dysfunction of contraction; (3) a production of free radicals initiated by autoxidation of catecholamines altered cardiac function [32]. By contrast, in our study, we observed a moderate increase of markers of ROS-induced damage. These results may be explained by the absence of heart failure symptom, the earlier detection of cardiomyopathy and the small number of patients.

Accordingly, $F_2$ isoprostane levels measured in blood and urine, although being considered as reliable markers of lipid peroxidation, remained within normal ranges. Cracowski et al. showed that the 8-epi$PGF_2\alpha$ level was increased in patients with severe heart failure [33]. In our study, patients without heart failure had no increased isoprostanes levels. It has been shown that isoprostanes are rapidly metabolized and excreted *in vivo* [34]. The liposoluble antioxidants, i.e. β-carotene, α-tocopherol and vitamin A, were also

Table III. Oxidative stress status in patients with cardiac involvement or cardiac and muscular involvement.

| | TBARS (μmol/l) | 8-epiPGF2α | | Carbonyla-ted proteins (nmol/mg protein) | TAS (mmol/l) | α-tocopherol (μmol/l) | Vitamin A (μmol/l) | β-carotene (μmol/l) |
|---|---|---|---|---|---|---|---|---|
| | | Plasma (pg/ml) | Urines (ng/mmol creatinine) | | | | | |
| Usual values | 0.60–1.20 | 40–100 | 50–100 | 0.55–0.90 | 1.30–1.90 | 20–37 | 1.5–2.6 | 0.20–0.80 |
| II 4 | 1.55 | 71 | 67 | 0.89 | 0.98 | 44.6 | 3.1 | 0.27 |
| II 11 | 1.45 | 114 | 136 | 0.91 | 1.14 | 27.2 | 3.0 | 0.14 |
| III 25 | 1.55 | 72 | 102 | 0.92 | 1.05 | 22 | 2.6 | 0.21 |
| II 6 | 1.75 | 65 | 143 | 3.02 | 1.12 | 37 | 2.6 | 0.57 |
| II 3 | 1.65 | 75 | 86 | 2.25 | 1.05 | 36.5 | 2.4 | 0.41 |
| III 24 | 1.8 | 80 | 102 | 2.92 | 1.00 | 35 | 2.5 | 0.35 |

Patients II 4, II 11 and III 25: isolated cardiac disease; Patients II 6, II 3 and III 24: cardiac and quadriceps disease; Usual values were obtained in 100 healthy subjects aged 20–45 years without cardiovascular risk factors (diabetes, smoking, hypertension, dyslipidemia).



Figure 2. Representative immunofluorescence and immunolocalization of NOS-I in human biopsies of proband with heart failure as previously described [47,48]. Translocation of NOS-I (green) to the sarcolemma; nuclear (blue); sarcolemma (red).

within usual values in our population. Lipid soluble antioxidants are not the only antioxidants involved in the antioxidant defenses, so that plasma total antioxidant status can be altered in affected patients as compared to healthy carriers. This may perhaps be due to deficiencies in other antioxidants (hydrosoluble antioxidants, extra cellular antioxidant enzymes). A generally accepted index of OS would have been the ratio between reduced and oxidized glutathione. Unfortunately, not enough biological material was available to perform this assay.

Noteworthy we should also consider that OS in the group of affected patients differed between patients, depending on their clinical status. Thus, in patients with cardiac and quadriceps muscle disease, plasma carbonylated proteins levels were markedly higher than in those with isolated cardiac disease. It should be noted that the latter had CP levels ranging in similar values as those of healthy carriers. Accordance to previous studies, patients with the same mutation display either cardiac involvement or both cardiac and muscular disease [11,35]. One hypothesis to explain this difference in phenotypic expression could be a modification of the OS status driven by redox sensitive gene expression. Mutation in lamins may be a target for OS as also suggested by findings in kidney cells where cysteine residues in lamins have been reported as targets of oxidative damage [36]. In this way, ROS may act as potent intracellular second messengers [37]. Oxygen radicals might lead to modifications of secondary and tertiary structures of proteins [38]. This hypothesis is also proposed in Alzheimer's disease pathogenesis: a protein that is uniquely oxidized in the plasma was shown to be much more susceptible to oxidation than the corresponding control protein when plasma was subjected to OS *in vitro* [39,40].

Some limitations of this study have to be underlined: (i) number of patients: only 2 patients of the third generation were studied. The remaining patients of these generation were younger (< 40 years) and the phenotypic status was not defined because neurologic disease occurs only later; (ii) OS was measured on peripheral blood samples. Under these conditions, measurements reflect the "dilution" of the OS in the whole body; (iii) we measured TBARS to detect lipid peroxidation, as oxygen radicals are able to attack polyunsaturated fatty acids in membranes and lipoproteins, leading to lipid propagation chains. TBARS assay constitutes a global evaluation of lipid peroxidation. As recently referred by Del Rio et al., plasma MDA or TBARS concentrations obtained with the methods developed from 1970 to 1995 vary in a very wide range (from 0 up to 50 μmol/l) [41]. However, in our study, plasma TBARS concentrations given as usual values were in the range 0.60–1.20 μmol/l, which is in agreement with a correct assessment of this marker. Nevertheless, the measure obtained by the TBARS assays gives an idea of sample oxidizability, rather than sample oxidation. This perhaps constitutes an attempted explanation for the discrepancy between TBARS and isoprostanes levels in our study. Furthermore, free radicals may also promote protein oxidation in membranes [42]. The determination of carbonylated proteins had some advantages as compared to lipid peroxidation products: oxidized proteins appear earlier and are generally more stable [43,44].

With regard to NOS-II activity, only few studies have been performed on human myocardic NOS and mostly in patients with heart failure (NYHA III–IV class). They showed variable results [45–47]. Moreover, in previous studies, patients were at NYHA III or IV class, which differs from our study on patients classified in NYHA I–II class. The discrepancy in the NOS-II activities observed in our patients could therefore be explained by their clinical status (NYHA class I or II). Finally, NOS-I-derived ·NO may play a role in the autocrine regulation of myocardial contractility in heart failure, a finding also previously reported [48,49]. This potentially altered signalling activity and targets of NOS-I resulting from its translocation to the sarcolemma, may be important in the pathophysiology of cardiac dysfunction.

This study reports the possible implication of OS in an amyotrophic quadricipital syndrome with cardiac involvement associated with a lamin A/C mutation without heart failure. There we show for the first time that ROS production occurs early, before heart failure symptom develop. Also, a different OS profile is observed, depending on the phenotypic expression of the disease. This slowly progressive increase in ROS could be explained by the earlier detection of cardiomyopathy. We suggest that this modification of OS could be taken into account in the variable phenotypic expression of the disease. Alterations in phenotypic expression of the disease could thus be in relationship with a different redox-sensitive gene expression.

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