"Oxidative stress" and muscle aging: influence of age, sex, fiber composition and function

Barbara Marzani, Orietta Pansarasa and Fulvio Marzatico

Department of Physiological and Pharmacological Sciences, University of Pavia, Pavia, Italy.

Abstract

Aim of this research was to study human muscle aging and the influence of oxidative stress correlated with physiological factors (age, sex, fiber composition and function) by measuring the antioxidant enzymes activities: total and mitochondrial superoxide dismutase (total and MnSOD), catalase (CAT), glutathione peroxidase(GSHPx) the levels of glutathione and glutathione disulfide (GSH and GSSG) and redox index. We also measured the lipid peroxide amount . As about age-related changes, we studied 120 samples (18-91 year-old) and we noted the presence of a correlation between age and ROS-mediated damages. Futhermore, it seems that 65 years could be the age at which ROS-dependent damage becomes crucial and begins to show up. Our data about sex-dependent changes showed how males may be potentially more vulnerable to oxidative damage than females. Study about fiber composition in old group (65-90 year-old) reported that subjects with +40% type II fibers not only have lipoperoxide levels lower but also detossifing system against superoxide anion more efficent than –40% type II fibers group. We also study aging process in muscle with different functions ("active": characterized by rapid and coordinated changes during contraction, "inactive": generating low levels of tension and maintaining this for a great duration). We observed that ROS-mediated oxidative mechanisms are different in "inactive" vs. "active" muscles.

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The nature of mechanisms underlying aging is at present not well understood. Aging is a complex process involving morphologic and biochemical changes in single cells and in the whole organism.

Various theories are advanced about the possible causes of aging; many of which agree and each of which is supported by scientific evidence [1], one of these focuses the attention on oxidative damage induced by reactive oxygen species (ROS) [2].

It has been suggested that aging could be caused by the accumulated deleterious effects of ROS throughout the life span [3]. ROS production is a process that stimulates several defensive enzymatic and nonenzymatic systems. However, a small quantity of ROS escapes elimination by antioxidant defenses and survives to damage proteins [4,5], lipids [6,7], nucleic acids [8,9] and carbohydrates [10]. The amount of ROS increases during aging as a result of functional decline of the mitochondria [11,12]. Multiple damage may explain the decrease in respiration rate and in the activities of different respiratory chain complexes in human skeletal muscle [13,14].

The skeletal muscles are particularly vulnerable to oxidative stress because they are constituted in postmitotic cells which are liable to accumulate oxidative damage over time and they use a large amount of oxygen.

The effects of aging on skeletal muscle antioxidant systems may be quite different from those in liver, kidney, brain and heart [15]: recent evidence reported a marked enhancement in rat muscle antioxidant enzymes in old age [16]; however, few data are available on human skeletal muscle aging.

There is also significant difference between sexes in aging. Rikans and coauthors [17] reported that cytosolic superoxide dismutase (CuZnSOD) and glutathione peroxidase (GSHPx) activities displayed sex-dependent variations in rat liver.

In human aging there are also sex defferences: in women estrogens protect cultured neurons against lipid peroxidation induced both by $FeSO_4$ and amyloid â–peptide [18]. Recently, it was also demonstrated the antioxidant effect of estrogens against cardiovascular diseases [19], hepatic fibrosis [20]

Table I. Inclusion and exclusion criteria used

Inclusion criteria	Exclusion criteria
Simple bone lesions Cysts, lipomas, varicose veins Surgical operations for mild pathologies: crural and internal hernias Ovariectomies for cysts Hysterectomy due to myoma Colecystectomy due to calculosis Cysts and lipoma of the abdominal wall	Fractures affecting neurological or muscular functions Peripheral neuropatheis Myopathies Rheumatoid arthritis Severe cardiopathies Malignant cancer Diabetes

At the cellular level, muscle aging determines cross-sectional area decline, fiber denervation and fiber number loss, mainly of type II fibers [21,22]. Type II fibers show an age related reduction in both number and size [23]; on the other hand, type I fibers increase with aging or are little affected [24,25,26]. The combination of these findings indicates a large proportion of the age-dependent loss in muscle mass as the result of the decline in type II fiber size [27].

With any reported body changes it must be considered whether neuromuscular responses are also a natural outcome of aging or simply a reflection of the age-related decline in physical activity.

In a review on muscle fiber type transitions Pette and Staron [28] suggested that age-related changes may be muscle specific.

Regarding a possible heterogeneity of age-related changes in muscle and the involvement of muscles in different functions, it seems appropriate to compare the aging process in functional separate muscles [29].

In this research we analyzed human muscle aging and the influence of oxidative stress correlated with physiological factors: age, sex, fiber composition and function.

We measured the superoxide anion (O_2) dismutating capability (total SOD and mitochondrial SOD), hydrogen peroxide (H_2O_2) detossifing system: catalase (CAT) and GSHPx, the reducing capacity of cell: levels of glutathione and glutathione disulfide (GSH and GSSG) and redox index. We also measured the lipid peroxide (LPO) amount as a marker of lipids damage.

Materials And Methods

Chemicals

Unless otherwise specified, all chemicals and reagents used were obtained from Sigma Chemical Co. (St Louis, MO, USA) and were of analytical grade or the highest grade available.

Subjects

Table II. Samples used in this study and their division in "active" and "inactive" group.

Active:	Inactive:
vastus lateralis peroneus longus sartorius tibialis anterior rectus femor gluteus maximus gastrocnemius adductor magnus	rectus abdominus latissimus dorsi supraspinatus external obliquus internal obliquus

Human muscle samples (n=750) were obtained with informed consent from patients hospitalized for various reasons. On the whole, men and women who took part to this study were in a healthy state. The inclusion clinical criteria are summarized in Table I. The total weight of each muscle sample obtained during the surgical procedure was approximately 3 g, of which we used 150–200 mg.

Age. For our evaluations we employed 120 samples used to carry out morphologic, morphometric, ultrastructural, biochemical and biomolecular evaluations. Muscle samples were substantially of three types: the vastus lateralis, the rectus abdominis and the gluteus maximus. There were 57 male and 63 female samples; the individuals were divided into eight groups: 18–25-year-old group (n=8, 4 men and 4 women); 26–35-year-old group (n=8, 5 men and 3 women); 36–45-year-old group (n=10, 6 men and 4 women); 46–55-year-old group (n=25, 10 men and 15 women); 56–65-year-old group (n=22, 13 men and 9 women); 66–75-year-old group (n=25, 11 men and 14 women); 76–85-year-old group (n=15, 6 men and 9 women); and 86–91-year-old group (n=7, 2 men and 5 women).

Sex. Of the 120 samples 57 were from men and 63 were from women. Samples was divided into three groups according to the age of the donor: young (18-40, n=19, 11 men and 8 women), adult (41-65, n=53, 26 men and 27 women) and aged (66-91, n=48, 20 men and 28 women).

Fibers.

We reported [31] that the average composition of type II fibers was of about 50% in subjects less than 65 year-old, while in elderly individuals (over 65 year-old) it fell to 40%. Of the 45 samples obtained from elderly people 65–90 year-old, 17 were from men and 28 were from women. We divided our samples into two groups according to type II fiber characterization: one group (n=25) with more than 40% type II fibers (+40% fibers II) and one group (n=20) with less than 40% type II fibers (-40% fibers II). In order to distinguish the type I and II fibers we used a histochemical analysis of myosin ATPase. Muscle tissue samples were pre-incubated under different acid (pH 4.4) and alkaline (pH 9.6) conditions. In general, slow myosins (type I fibers) are

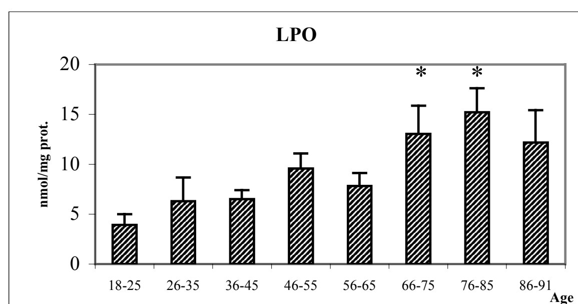


*Fig. 2. Comparison of LPO levels in skeletal muscle of subjects of different ages. Values are expressed as mean ± SEM. *p<.05 vs. 18-25-year-old group.*

strongly dyed at pH 4.4 and inactivated at pH 9.6, while fast myosins (type II fibers) are highly dyed at pH 9.6 and inactivated at pH 4.4 [30]. The ratio between type I and II fibers in over 65 year-old individuals is 1.56, while the same ratio in individuals under 65 year-old is 1.01 [31].

Function.

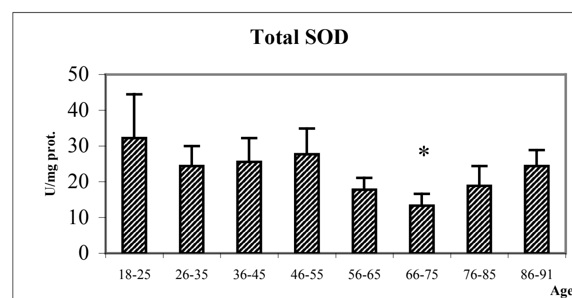
57 subjects participated in this study. Neuromuscular or other chronic diseases, which are known to lead to changes in the muscle and diseases that considerably impair motor activity, were excluded.

We divided our samples into active and inactive muscles (Table II).

The division criteria is the follow: "active" muscles have neuromuscular system with rapid nerve conduction and they are characterized by rapid and coordinated changes during contraction, "inactive" ones have neuromuscular system with slow nerve conduction and generate low levels of tension, however they are able to maintain this for a great duration.

Tissue preparation

Skeletal muscle samples used to detect total SOD, GSHPx, CAT activities and LPO content, were weighed and diluted 20% w/v in 20 mM TRIS–HCl, pH 7.4. Samples were minced, homogenized with a Polytron tissue processor and subsequently homogenized in a pre-cooled Potter–Braun homogenizer. Part of the homogenate was used to detect LPO content, while the remaining fraction was centrifuged at 5000 x g for 10 min in a refrigerate centrifuge (Beckman J21C) and then, the supernatant fraction, was used to measure total SOD, GSHPx and CAT activities. MnSOD activity was evaluated on the mitochondrial fraction obtained after two homogenizations, once with a Polytron and the second time with a precooled Potter–Braun, in 0.32 M sucrose and 1 mM ethylendiaminetetracetate (EDTA) buffer, and then centrifuged at 800 x g for 15 min (4°C Beckman J21C centrifuge). Supernatants were centrifuged once more at 14000 x g for 20 min (4°C Beckman J21C centrifuge), and the mitochondrial pellet was resuspended in sucrose–EDTA (final dilution 10 mg/0.1 ml). To evaluate glutathione levels, muscle samples were weighed, diluted 10% w/v in 5% sulfosalicilic acid (SSA), homogenized in a Polytron and in a precooled Potter–Braun, and then centrifuged in a refrigerate centrifuge (Beckman J21C) at 9500 x g for 5 min. The resulting supernatant was used for the measurements.



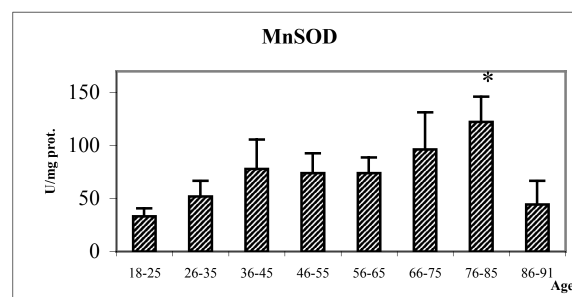


*Fig. 1. Effect of aging on total SOD (a) and MnSOD (b) activity in skeletal muscle of subjects of different ages. Values are expressed as mean ± SEM. *p<.05 vs. 18-25 year-old group.*

Biochemical determinations

Total (10 mM KCN) and MnSOD (1 mM KCN) activities were evaluated by monitoring at 550 nm the rate of cytochrome c reduction by O_2•- generated by the xanthine/xanthine oxidase system [32]. CAT activity was measured spectrophotometrically at 240 nm following the H_2O_2 decay [33]. GSHPx activity was detected measuring at 340 nm the NADPH oxidation in the presence of both GSH and H_2O_2 [34]. Total GSH (GSHtot) levels were assayed by means of the 5-5'-dithionitrobenzoic acid (DTNB) cyclic reaction, monitored at 412 nm [35]. The oxidized form (GSSG) was measured according to the same method after derivatization of GSH with 2-vinylpyridine. The LPO content, i.e. malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), was evaluated colorimetrically at 586 nm according to Esterbauer and Cheeseman [36]. Sample protein concentration was measured using serum bovine albumine as a standard [37].

Statistical analysis

Statistical analysis was carried out with the one-way (ANOVA) analysis of variance and Fisher's test for multiple comparison. Differences were considered statistically significant at p<.05.

Results

Age. As regards the age-dependent changes the most important results are the follow:

Total SOD and MnSOD activity

Age-dependent changes in total SOD activity are shown in Fig. 1a. We observed a significant decrease in the 66–75 year-old group ($p<.05$) with respect to the control group (18-25 year-old). On the other hand, MnSOD activity increases significantly in the 76–85 year-old group vs. control (Fig. 1b).

GSHtot, GSH, GSSG levels and redox index

The age-related changes in GSHtot, GSH, and GSSG levels are reported in Table III. It was observed that GSHtot and GSH levels did not change with age, although a significant increase in GSSG levels was observed for the 76–85 and 86–91 year-old groups ($p<.05$). Table III also reports the age-dependent changes in cell redox state expressed by the redox index: ([GSH] + 2[GSSG])/(2[GSSG]*100). No significant changes were observed.

Levels of LPO

Lipid peroxidation levels are rapresented in Fig. 2. A significant increase (p<.05) in LPO levels was observed in the 66-75 and 76-85 year-old groups compared with the control group.

Sex.

Levels of LPO

A significant lower level was observed in young women vs. their male counterpart (Fig. 3).

GSH, GSSG levels and redox index

GSH levels remained unchanged between the two sexes. As regards GSSG we observed significant lower levels in 66-91 year-old women vs. 66-91 year-old men. The redox index was significantly higher in young and aged women vs. young and aged men respectively (Table IV).

Fiber.

Total SOD and MnSOD activity

As reported in Table V, total SOD activity is lower in the -40% fibers II group (p<.05) than in the +40% fibers II group. MnSOD activity tended to be lower, but without gaining the statistical significance.

LPO levels

A significantly higher level (p<.05) of LPO was observed in the -40% fibers II compared to those with +40% type II fibers (Fig. 4).

Function.

MnSOD activity

We observed an age-dependent MnSOD increase both in “active” and “inactive” muscles particularly evident in “inactive” ones (p<.05 18-45 vs. 66-90) (Fig. 5a).

Age group (years)	*GSHtot* (nmol/g tissue)	*GSH* (nmol/g tissue)	*GSSG* (nmol/g tissue)	*Redox Index*
18–25	805.57 ± 26.25	754.51 ± 21.32	51.07 ± 16.19	0.093 ± 0.023
26–35	789.39 ± 127.86	737.51 ± 132.35	51.88 ± 12.40	0.092 ± 0.023
36–45	944.48 ± 108.82	920.34 ± 119.91	40.68 ± 16.50	0.210 ± 0.062
46–55	699.69 ± 118.85	553.47 ± 112.21	62.97 ± 15.23	0.080 ± 0.021
56–65	695.93 ± 85.21	628.99 ± 88.94	74.32 ± 16.98	0.082 ± 0.015
66–75	741.80 ± 122.47	619.14 ± 110.97	122.65 ± 32.54	0.066 ± 0.019
76–85	1084.84 ± 178.38	823.61 ± 190.94	261.23 ± 137.38*	0.077 ± 0.033
86–91	1186.96 ± 150.94	882.46 ± 277.10	304.5 ± 169.92*	0.047 ± 0.027

*Table III. Age-related changes in GSHtot, GSH, and GSSG levels and the redox index, ([GSH] + 2[GSSG])/(2[GSSG]*100), evaluated in the eight age groups. Values are expressed as means ± SEM.*

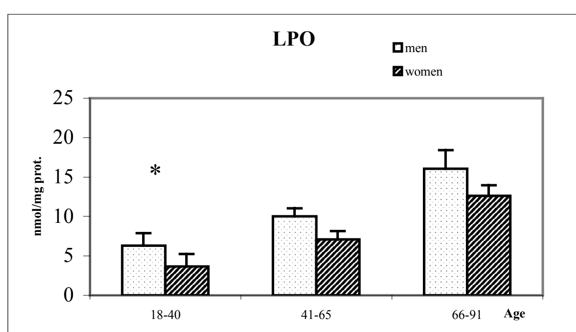


*Fig. 3. Sex differences on lipid peroxide levels in skeletal muscle of men and women in three experimental groups. Values are expressed as mean ± SEM. *p<.05, 18-40 year-old men vs. 18-40 year-old women.*

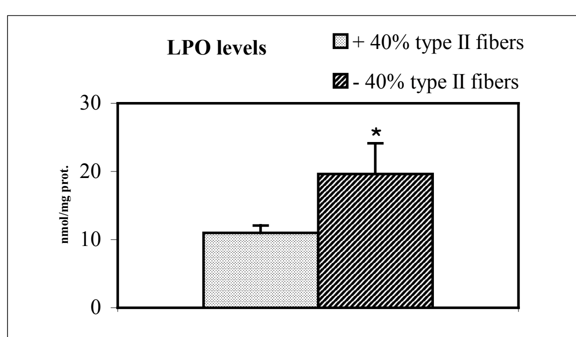


*Fig. 4. Type II fiber-dependent changes in lipid peroxide (LPO) levels, evaluated in +40% type II fibers and in -40% fibers of 65–90 year-old subjects. Values are expressed as means ± SEM.*p<.05 when comparing -40% vs. +40% type II fibers.*

LPO levels

LPO levels rapresented in Fig. 5b reported an increase during aging, moreover the process acts differently into two types of muscles, infact it is more marked in "active" ones with a significant increase ($p<.04$) between 18-45 vs. 66-90 year-old group.

Conclusion

Aging is a process characterized by several changes that include a reduced capacity to use oxygen along with impaired cardiocirculatory capacity and respiratory adaptation; deterioration of the nervous system: a decrease in the form, width and rate of conduction of evoked potential; and degeneration in muscle mass characterized by a reduction in muscle fiber diameters and by a qualitative and quantitative alteration in muscle fibers.

The age-dependent SOD activity changes in the skeletal muscle are debated: some experimental studies shown an age-dependent increase in total SOD activity [38]; other researchers report that there are no changes in total SOD activity, but find an increase in MnSOD activity in different rat tissues [39].

Our results indicate that in human skeletal muscles there is a significant decline in total SOD activity during aging and a significant increase MnSOD activity. The increase in MnSOD activity obtained in our study agrees with that reported by Ji et al. [16]. Their data indicate the existence of regulatory mechanisms in senescent tissues which provide an induction of the defenses against O_2•- that may be generated at a higher rate in mitochondria during aging.

In our study, we observed that skeletal muscle GSHtot and GSH levels remained unchanged with age indicating that membrane transport of GSH into the muscle cell, which plays a critical role in determining tissue GSH levels, did not change during aging [40]. Even if we observed a significant increase in the oxidized form of glutathione during aging, it is unable to decrease significantly the cell redox state [41], reported as the redox index, which remains relatively constant during senescence. Aging could cause alterations of glutathione status in human skeletal muscle, but it is difficult to demonstrate these alterations because of the heterogeneity of the muscle fiber type that we used.

An interesting possible explanation for the age-dependent reduction of membrane fluidity is the oxidation of lipidic components of the membrane. Membranes and lipids are particularly susceptible to the oxidation process [42,43] and to the peroxidative reaction induced by ROS and catalyzed by transition metals such as iron and copper [44,45]. There are few data concerning age-dependent changes in LPO levels in humans during aging: in plasma a small but significant increase was observed with increasing age [45]. With regard to muscle LPO, Mecocci et al. [46] demonstrated that there is an age-dependent increase in MDA content. The data obtained in our research agree with those reported by these authors.

Moreover, from our data it seems that about 65 years could be the age at which ROS-dependent damage becomes crucial and begins to show up.

No significant changes were observed in GSH levels between the sexes. However we noted that men have lower GSH levels than women in all the three experimental groups. On the other hand, aged women have lower GSSG levels than aged men. An age-dependent alteration in the glutathione status is supported by the fact that the redox index is significantly higher in 18-40 year-old women than in male counterpart; the same results were obtained in aged women vs. aged men.

Lower LPO levels were observed in young women vs. young men, consistent with the protective effect of female sexual hormones against lipid peroxidation.

Our data show high LPO levels both in aged men and aged women: the two sexes show the same

susceptibility to peroxidation because of the failure of the protective role of estrogens after menopause during female aging, which makes aged women more sensible to LDL oxidation and to cardiovascular disease than aged men [18].

So it seems that men are more susceptible than women to age-dependent in the oxidative status of skeletal muscle.

An other aspect that we must consider is fiber composition infact in the present study, where we examined only elderly people (65-90 year-old), we observed a significantly lower total SOD activity in the -40% fiber II group than +40% type II fiber; our data showed the same tendency in MnSOD activity.

Probably, the decline in total superoxide scavenger capability observed in elderly subjects, becomes more evident when the percentage of type II fibers decreases further. Thus, it seems that muscles with high type I fiber percentage are more vulnerable to ROS-mediated damage, not only because they have a large number of mitochondria, but also because age-related mutations in mtDNA could interfere with cellular ATP production causing interference in the respiratory chain and as a consequence a reduced O_2•- dismutating capability [47].

As previously stated [31,46,48] LPO levels are significantly higher in aged subjects than in young subjects. In this study, we reported a two fold increase in LPO levels in aged individuals with -40% fiber II compared to those with +40% type II fibers. This suggests that the increase in lipid peroxidation, a representative marker of oxidative damage, prevails in the type I fibers where most ROS production probably takes place.

Hence, skeletal muscles of individuals with a high percentage of type I fibers are also more vulnerable to ROS-mediated damage.

However, it was unquestionably necessary to consider the age process in different types of muscle which differ for function.

The enhance in MnSOD activity is age-dependent and it is particularly evident in "inactive" muscles ($p<.05$ 18-45 vs 66-90) where it could be hypothesized a marked alteration of electron transport chain functionality with a consequent leakage increment.

With regard to muscle LPO, the data obtained in our research agree with those reported by others authors [45, 46] but the increase in LPO levels are more evident in the "active" muscles. It seems to suggest a muscle-specificity correlation.

In this case we are not able to indicate a reason of this, but we could to hypothesize that the LPO increase are the final effect of a additive situation related to major contractile function operated by these muscles or it may be due to composition problem: these muscles have more lipid peroxidable substrates. So appears that aging does not affect in the same manner LPO; aging process may be different with regard to different substrates.

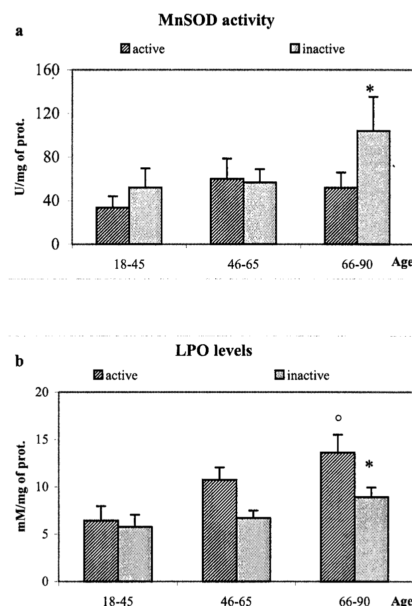


Figure 5. Aging and muscle differences on MnSOD activity (a), lipid peroxidation levels (b) in "active" and "inactive" skeletal muscle in three experimental groups: 18-45, 46-65 and 66-90 years old.Values are expressed as mean ± SEM.
**p<.05, 18-45 "inactive" muscle vs. 66-90 "inactive" ones.*
°p<.04, 18-45 "active" muscle vs. 66-90 "active" ones.

In breaf, our data are consistent with evidence of the involvement of oxidative damage in the aging muscle process futhermore muscle-function differences seem to influence aging-related changes in specific way in different muscles.

The results of this study, the first study of this kind to be conducted on such a great number and age range of human muscle samples, suggest that there is a correlation between ROS activity and physiological factors such as age, sex, fiber composition and function.

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Address correspondence to:

Fulvio Marzatico, Chief of Farmacobiochemistry Lab, Department of Physiological-Pharmacological Cellular and Molecular Sciences, Section of Pharmacology and Pharmacological Biotechnologies, University of Pavia, P.zza Botta 11, 27100 Pavia, Italy, Tel.+39-0382-506390, Fax +39-0382-506390
e-mail: fulviomk@unipv.it

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