DISCUSSION

Increased oxidative stress has emerged as playing a central role in metabolic syndrome.[225-228] Oxidation is a chemical process whereby electrons are removed from molecules.[229] Molecular oxygen is the final electron acceptor for cytochrome c oxidase (complex IV, the terminal component of the respiratory chain) and is ultimately reduced to water. However, a small quantity of oxygen may be incompletely reduced as a leakage of single electrons causes the reduction of oxygen to superoxide ($O_2^{\cdot-}$) and highly reactive free radicals are generated. These free radicals include reactive oxygen species (ROS). The excess of ROS can damage cellular lipids, proteins and DNA.[230]

The delicate balance between beneficial and harmful effects of ROS is a crucial aspect in living organisms, and is achieved by mechanisms referred as "redox regulation". This process protects living organisms from different forms of oxidative stress.[231] It is conceivable that mitochondria are more vulnerable to oxidative damage than other cellular organelles.[232]

The ability to utilize oxygen has provided humans with the benefit of metabolizing fats, proteins and carbohydrates for energy. Mitochondrial respiration consists of the oxidation of the main substrate (NADH) supplied by glucose, fatty acids and amino acids, which is coupled to the pumping of protons across the mitochondrial inner membrane. The ATP synthase, inside the mitochondrial inner membrane, couples the transport of the protons across the membrane to the synthesis of ATP inside the mitochondria. Thus mitochondria convert energy stored in nutrient into ATP/ADP that drives work within the body.[233]

In physiological conditions cells defend themselves against increase ROS through antioxidants that remove free radical intermediates and inhibit oxidation. Intracellular signaling effectors, including H_2O_2 reflects the balance between the electron leak/superoxide formation from the respiratory system and scavenging of H_2O_2 across diverse forms of aerobic life.[234]

Imbalance between endogenous oxidants and antioxidants results in oxidative stress.[235] Also the oversupply of substrates by overnutrition generating surplus reducing-equivalents could in turn expected to elevate the redox state increasing oxidative stress.[236] **James et al (2012)**[237] explained that under conditions of overnutrition, excess electrons supply to the respiratory chain, while lack of physical activity (low ATP demand) typical to those that foster the metabolic syndrome, will favour ROS formation.

In the present work, we focus on oxidative stress events leading to individual disease factor appearance in metabolic syndrome patients. An increase of more than 100% of an oxidatively damaged guanine (8-OHdG) produced in DNA damage, due to ROS, in metabolic syndrome patients, compared to healthy control subjects was obtained. This was in agreement with **Hutcheson and Rocic (2012)**[226], who stated that increased oxidative stress has emerged as playing a central role in metabolic syndrome. Many studies also suggested that elevated mitochondrial oxidative stress is frequently associated with, and may contribute to, the metabolic syndrome.[193,236,238-241]

Anderson et al (2009)[236], explained that when cells are in metabolic balance, substrate uptake and catabolic rates, through the metabolic pathways are matched to energy demand, ensuring that reducing equivalents (i.e. NADH, $FADH_2$) are provided at a rate sufficient to support respiratory demand. However, mounting evidence suggested that when cells are out of metabolic balance, the oversupply of substrates has a demonstrable impact on cell function. The generation of surplus reducing equivalents would in turn be expected to elevate the redox state of complex I of the respiratory chain.[242-245] Once in the cytosol, H_2O_2 can alter the redox state of the cell either by reacting directly with thiol residues within redox-sensitive proteins or shifting the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG), the main redox buffer of the cell. Thus, the rate at which H_2O_2 is emitted from mitochondria is considered an important barometer of mitochondrial function.[246]

Anderson et al (2009)[236] reveal that the muscle redox environment, as reflected by both GSH/GSSH ratio and total cellular glutathione content (GSHt), is remarkably sensitive to nutritional intake, shifting to a more oxidized state in response to both acute (e.g. glucose ingestion) and chronic (e.g. high-fat diet) food ingestion. They found a near doubling of GSSG and approximate of 50% reduction in (GSH/GSSG) ratio in muscle of both control and high-fat diet-fed rats within 1hour after oral glucose gavage. This is consistent with insulin stimulating glucose uptake and flux through metabolism, generating an increase in mitochondrial H_2O_2 emission that is in turn buffered by GSH. They added also that the response to carbohydrate ingestion is transient, owing to the rapid clearance of glucose, and dietary lipids may elicit a more sustained elevation in H_2O_2 emission, shifting the cellular redox environment to a more persistent oxidized state. **Domenicali et al (2005)**[247] added that increased generation of mitochondrial ROS and oxidative damages seem to be differently induced by nutritional perturbation and state.

Metabolic abnormalities in our metabolic syndrome patients were defined by the presence of abdominal obesity (increased waist circumference), insulin resistance along with its associated hyperinsulinemia, elevated triglycerides and fasting glucose, low high-density lipoprotein and high blood pressure. We obtain linear combinations of these variables including (waist circumference, hypertension, hyperglycemia, dyslipidemia, and oxidative stress markers (8-OHdG and MDA). Abdominal obesity is consistent with increased oxidative stress markers in our results. These considerations are consistent with other metabolic syndrome factors, representing the trigger for systemic oxidative alterations.

This agree with **Grattagliano et al (2008)**[248] who suggested that accumulation of fat in the abdominal region, and that in the liver in particular induce increases in systemic lipid peroxidation and damages through excess free fatty acids (FFAs), lipoprotein-bound lipids, cytokines and vasoactive peptides. Thus, these considerations are consistent with abdominal fat and liver steatosis as the initial alterations responsible for the subsequent appearance of other metabolic syndrome factors, representing the trigger for systemic oxidative alterations and revealing a purported incapacity of patients to correct excess oxidation. Also, **Perticone et al (2001)**[188] stated that obese patients have shown oxidative stress-induced decreased vasodilatory response to acetylcholine, which was inversely related to body mass index, waste to hip ratio, fasting insulin and insulin resistance.

High serum oxidative stress markers obtained in this study (8-OHdG, MDA) correlate positively with elevated fasting glucose, HOMA-IR, triglycerides, LDL and inversely with HDL and SOD. Similar results were obtained in a group of metabolic syndrome patients with end stage renal disease.[226]

Also **Oliveira et al (2011)**[195] found that LDL receptor-deficient mice fed a cholesterol-enriched diet developed elevated LDL levels and consequently oxidative stress. These observations extend to human studies. Similar results were achieved by **Zelzer et al (2011).**[197]

We can say that the positive correlations obtained in the present results between hyperglycemia, dyslipidemia, IR and 8-OHdG may suggest a role of increasing intracellular metabolism and consequent increase in ROS production. This agrees and is explained by the following studies:

Davi et al (1999)[249]**, Nishikawa et al (2000)**[250] and **Bonnefont-Rousselot (2002)**[251] explained that the increased intracellular metabolism of glucose in the hyperglycemic states leads to nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) overproduction with consequent stimulation of adenosine triphosphate (ATP) generation by the electron transport chain. Excess NADH causes increases in the mitochondrial proton gradient and, as a consequence, electrons are transferred to oxygen, producing superoxide.

Different studies explained the molecular integration between lipid and glucose. **Donohoe et al (2011)**[252] stated that since the levels of adipose tissue affect the body's handling of glucose, the high concentration of cytokines produced by adipose tissue, such as TNF-α, IL-6, IL-1β and low concentration of adiponectin, may have deleterious effects on glucose homeostasis leading to chronic hyperinsulinemia and insulin resistance. Also **Jensen (2008)**[253] and **Despres (2012)**[254] stated that individuals with excess visceral fat are at higher risk of developing insulin resistance in the metabolic syndrome. **van Kruijsdijk et al (2009)**[255] agree also with the molecular integration between lipid and glucose metabolism, they stated that high levels of FFAs, as seen in obesity, reduce insulin-mediated glucose uptake by the GLUT4 transporter and inhibit the insulin receptor-mediated tyrosine phosphorylation of the insulin-receptor substrate-1 (IRS-1).

However **Hardie (2008)**[256] has focused on reversing oxidative stress. He suggested that the metabolic syndrome involves a cluster of related metabolic abnormalities that are all potentially reversed by 5'-adenosine monophosphate-activated protein kinase (AMPK) activation, which inactivate key enzymes of lipid biosynthesis. It is a key player in the regulation of the balance between whole-body energy intake and energy expenditure, and hence in the development of obesity. He explained also that activation of kinases in response to exercise or to pharmacological agents such as (biguanides) can activate AMPK in part by inhibiting Complex I of the respiratory chain, and thus elevating cellular AMP/ATP ratios, reversing the metabolic abnormalities associated with type 2 diabetes and metabolic syndrome.[257,258]

Present results also link positively oxidative stress and hypertension, this agree with **Redon et al (2003)**[201] and **Wang et al (2009)**[202]. Furthermore, **Sanchez et al (2007)**[206] stated that hypertension was found to be secondary to IR. Oxidative stress has been shown to increase with deoxycorticosterone acetate (DOCA)-salt-induced hypertension,[208] and

with angiotensin II infusion,[209] as well as in genetic animal models of spontaneous hypertension (SHR).[259] **Reaven et al (1996)**[260] also suggested the participation of arterial hypertension in the generation of systemic oxidative stress associated with the metabolic syndrome. On the contrary, a study in metabolic syndrome patients showed that other metabolic syndrome components (low HDL, abdominal obesity, TG and fasting glucose) had minimal contribution to increased oxidative stress, whereas hypertension alone was responsible for elevated oxidative stress in these patients.[203] It is not clear how the effects of hypertension were separated from the effects of the other risk factors in this study.

In the present study we obtained a positive correlation between oxidative stress markers (8-OHdG& MDA) and IR in our metabolic syndrome patients. This agrees with **Bonnard et al (2008)**[240] and **Houstis et al (2006)**[193] who concluded that oxidative stress may be a major determinant in the loss of both insulin sensitivity and mitochondrial function associated with high dietary fat intake and obesity. **Evans et al (2005)**[261] also stated that ROS accumulation in general is associated with cellular insulin resistance, and this can occur through activation of stress kinases or injury to cellular membranes, the endoplasmic reticulum and/or nuclear DNA. Oxidative modification of cellular proteins and lipids may have functional consequences that contribute to insulin resistance.[261] **Schmitz-Peiffer and Whitehead (2003)**[262] suggested that insulin receptor substrate (IRS) function rather than amount can be a critical mechanism for insulin resistance in 'metabolic stress' exerted by elevated insulin, glucose and possibly FFA levels. This is likely to be mediated via an altered phosphorylation status. This impairs the interaction with other signaling proteins such as phosphatidyl inositol 3-kinase, and it also appears to direct IRS-1 towards proteasome-mediated degradation. **Lundgren et al (2007)**[263] also stated that in 'overloaded' adipocytes, it is possible that an increase in intra-adipocyte fatty acid levels will be accompanied by oxidative stress. This may partly explain the strong link between fat cell enlargement and insulin resistance. Clinical data also suggested that overall oxidative stress is increased in subjects with visceral obesity.[264]

Bonnard et al (2008)[240] reported deteriorations in mitochondrial structure and function in mice skeletal muscle which appear only after several months of high-fat feeding, well after insulin resistance has developed. This implication is that mitochondrial dysfunction, similar to insulin resistance, is a consequence of the altered cellular metabolism that develops with nutritional overload.[236]

Thus, we can say that there is now much emerging evidence that suggest that several factors that cause insulin resistance have a common pathway in the excessive formation of ROS.[193,261,265] The same findings was obtained also by **Lowell and Shulman (2005)**[266] they found that insulin resistance abnormalities associated with elevated TG storage in tissues other than adipose tissue, such as muscle and liver, are in association with a relative defect in mitochondrial oxidative capacity in these organs.

In view of the evidence supporting a link between excess lipid accumulation and insulin resistance in muscle, several studies suggested that mitochondrial dysfunction represents the mechanism underlying the accumulation of lipid metabolites and the development of insulin resistance.[236] **Lowell and Shulman (2005)**[266] and **Morino et al (2006)**[267] stated that acquired or inherited mitochondrial dysfunction limits the capacity to oxidize fats in skeletal muscle. The same idea was supported by **Mootha et al (2003)**[268] and **Patti et al (2003)**[269] who stated that skeletal muscle of obese individuals is

characterized by profound reductions in mitochondrial function, as evidenced by decreased expression of metabolic genes, reduced respiratory capacity,[270-272] and mitochondria that are smaller and less abundant,[270] leading to the speculation that acquired or inherited mitochondrial insufficiency may be an underlying cause of the lipid accumulation and insulin resistance that develops in various metabolic states.[266,267]

Thus, we can conclude that oxidative stress plays a number of potential mechanisms in the pathophysiology of metabolic syndrome. We suggest also that several factors that cause insulin resistance have a common pathway in the excessive formation of reactive oxygen species.

The recognition of mitochondria as an arbiter in the life and death of cells has highlighted the need to develop antioxidants and other cytoprotective agents.[230] **Hutcheson and Rocic (2012)**[226] suggested that increased oxidant capacity by chronic overnutrition in the metabolic syndrome coupled with decreased endogenous antioxidant capacity results in oxidative stress.

In the present results a significant decrease in serum total SOD enzymes in metabolic syndrome patients compared to the healthy control group, suggests a decreased endogenous mitochondrial antioxidant capacity. Interest has focused for many years, on strategies that enhance endogenous antioxidant defense using either antioxidants.[273,274] We discuss in the present study the effect of antioxidant properties of some pharmacological agents including atorvastatin (followed-up for 3months), which is a synthetic lipid lowering agent,[275] and vitamin E, a major lipid soluble antioxidant,[276] on complications of metabolic syndrome.

Quantitative estimation of oxidative stress markers (8-OHdG& MDA) revealed improvements after atorvastatin therapy. A decrease of about 30% of an oxidatively damaged guanine (8-OHdG) produced from DNA-damage as a result of ROS suggests that atorvastatin possesses antioxidant properties. **Brea et al (2012)**[235] also have suggested that atorvastatin treatment affects oxidative stress marker levels. On the other hand, improvement obtained also in SOD enzyme may suggest enhancing endogenous antioxidant defense. Present results with atorvastatin treatment demonstrate also a highly significant reduction in both total cholesterol and LDL and show a significant increase in HDL. However atorvastatin fail in reaching therapeutic effect on both hypertension and fasting glucose levels.

We suggest that the beneficial effects of atorvastatin appear to be greater than that might be expected from changes in lipid levels alone. It is now accepted that numerous positive effects of some statins in the cardiovascular system are independent of their lipid lowering effect and as a consequence of a direct decrease in oxidative stress.[198] **Andreadou et al (2012)**[198] added that short-term pravastatin treatment reduced myocardial infarct (MI) size in hypercholesterolemic rabbits through reduction in peroxynitrate and nitrotyrosine formation. Statins can also restore endothelial function by increasing endothelial nitric oxide synthase (eNOS) expression, decreasing eNOS uncoupling, reducing $ONOO^-$ level (nitroxidative stress) and shifting the $[NO]/[ONOO^-]$ balance towards NO.[277]

In the present work atorvastatin elevated total SOD activity by more than 20%. This agrees with **Ansari et al (2012)**[199] who found that rosuvastatin lowered oxidative stress by elevating the expression of antioxidant enzymes (SOD, catalase, glutathione and glutathione peroxidase).

Liao and Laufs (2005)[278] explained that statins are a group of lipid-lowering drugs, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, used in the prevention and treatment of cardiovascular diseases. Statins also possess cholesterol-independent or "pleiotropic" effects, which include improvement of endothelial function, stabilization of atherosclerotic plaques, inhibition of oxidative stress and inflammation, and a reduction of thrombogenic response.[278] These beneficial effects of statins are, at least in part, mediated by an effect on endothelial nitric oxide synthase (eNOS).[279-281] But several statins also inhibit endothelial $O_2^{\bullet -}$ formation[282] by reducing the expression and/or activity of NADPH oxidase[282,283] leading to amelioration of oxidative stress.[284] These effects may be partly responsible for the antiatherogenic action of statins.[285-287] Since statins have effects on eNOS and NADPH oxidase, it is not surprising that statins treatment could influence levels of oxidative stress markers, such as 8-OHdG, MDA and SOD. Thus, present results suggest that oxidative stress markers levels correlate with outcomes in metabolic syndrome patients treated with atorvastatin.

We evaluate also in the present study the prognostic value of oxidative stress markers in a group of metabolic syndrome patients receiving combined treatment of atorvastatin+ vitamin E for 3 months. We report inverse association between oxidative stress markers before and after (atorvastatin+ vitamin E) supplementation. MDA was significantly decreased; however, this level of vitamin E supplementation did not alter 8-OHdG significantly, when compared with atorvastatin alone. Similar results were achieved by **Wu et al (2007)**[288], **Lagadu et al (2010)**[289] and **Garelnabi et al (2012)**[290].

Combined treatment with atorvastatin plus vitamin E had no effect on fasting glucose and hypertension, but had a beneficial effect on LDL, TG, HDL and SOD. Several studies (animal and human studies) found also that vitamin E intake blocks LDL lipid peroxidation, and prevents the oxidative stress linked to T2DM-associated abnormal metabolic patterns (hyperglycemia, dyslipidemia, and elevated levels of FFAs).[291-294]

Devaraj et al (2008)[295] suggested that markers of oxidative stress, such as MDA and lipid peroxides, were significantly reduced in metabolic syndrome subjects after vitamin E treatment, regardless of which isoform of vitamin E was used. They also suggested that supplementation with combined α and γ tocopherols may be more beneficial in reducing oxidative stress and inflammation than either isoform alone.[295] However, some studies using conventional antioxidants as ROS scavengers have failed in reaching therapeutic goals.[296]

We can conclude that in metabolic syndrome patients during aerobic metabolism, chronic overnutrition coupled with physical inactivity generating surplus reducing equivalents would in turn expected to elevate the redox state, increasing oxidative stress. Obtained improvement in the quantitative estimation of oxidative stress markers in metabolic syndrome patients after atorvastatin therapy suggests that atorvastatin possesses antioxidant properties by reducing lipid peroxidation and reactive oxygen species production.

Another interesting point, although it is clear that the major risk factors in metabolic syndrome are chronic overnutrition and physical inactivity, the interaction between genetic and environmental influences may have a role in increasing susceptibility to oxidative stress. According to several studies who reported also association between mitochondrial insufficiency and decreased expression of metabolic genes we may suggest that their role in this condition is supported by familial clustering. This would stimulate prospective studies dealing with the changes in oxidative parameters and appearance of metabolic abnormalities in family clusters.

Future research in the field of metabolic diseases should include elucidation of potential new mechanisms and pharmacological targets related to ROS production and accumulation.