Oxidation products of DNA, lipid and protein among the individuals progressing towards metabolic syndrome

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Oxidative stress (OS) is an early event and at the same time also a consequence in the pathology of MetS. We investigated if oxidation markers of DNA, lipid and protein increased with an increase in the risk parameters of MetS. Participants (male:70, female:90 $\geq$ 20 yrs) were categorized based on the number of risk factors they had as 3 Risk, 2 Risk, 1 Risk and 0 Risk for MetS and were evaluated for various oxidation markers. Protein carbonyl and advanced oxidation protein product (protein oxidation marker) differed significantly between the four study group while malondialdehyde and hydroxynonenal (lipid peroxidation marker) did not. "8-OH dG" (DNA oxidation marker) differed significantly ($P < 0.05$) while total antioxidant capacity did not demonstrate significant difference in its values across the group ($P > 0.05$). Pairwise comparison for statistically significant markers(Protein oxidation markers and 8-OH dG), demonstrated that only 8-OH dG differed significantly between 0 Risk- 3 Risk ($P < 0.012$) but not between 0 Risk -2 Risk and 0 Risk-1 Risk. Oxidative stress markers of DNA, lipid and protein do not increase with an increase in the risk parameters of MetS. However, it is indeed high in MetS with 3 and more risk parameters. Presence of 2 or 1 Risk also increases OS compared to 0 Risk. There is oxidative stress damage in MetS to lipid and protein but DNA damage was of significant consequence.

Keywords: DNA damage, Lipid peroxidation, Metabolic syndrome, Oxidative stress, Protein carbonyl

Aerobic respiration is obligated to generate reactive oxygen species (ROS) in the body. The generation of small amounts of ROS and free radicals play important roles in cellular signaling processes essential for a normal functioning of the human body[1]. However, when the ROS production supersedes the cellular capacity to detoxify them by antioxidants due to an unfavorable environment, the subtle balance between oxidation and antioxidation is lost and oxidative stress (OS) ensues. OS is an early event and at the same time also a consequence in the pathology of MetS which is a cluster of cardiovascular disease risk factors in one individual comprising of diabetes or pre-diabetes, abdominal obesity, high cholesterol and high blood pressure, all recognized easily by simple clinical measures. ROS of vascular origin plays an important causal role in the development of obesity *via* NADPH oxidase[2]. A robust number of studies also demonstrates that all the five classical risk factors of MetS increase the production of ROS. Poor diet and a lack of physical activity which are the risk factors of MetSare the

internal sources for OS. High caloric intake, composed of glucose, lipid, or protein beyond the body's energy requirement causes an increase in the generation of ROS by Leukocytes, p47phox protein, a key protein in the enzyme NADPH oxidase, activation of nuclear factor-κB and inflammation[3-6]. Different macronutrient induces a distinct pattern of increase in ROS generation[7]. OS builds up to damage macromolecules and targets of oxidative damage are DNA, protein, lipid and carbohydrate[8]. 8-hydroxyguanine, carbonyls, malondialdehyde and, advanced glycation end products are the few examples of oxidative damages to the bio-molecules, respectively. ROS leading to protein oxidation include radical species such as $O_2^{\cdot -}$, $OH^{\cdot}$, peroxyl ($RO_2^{\cdot}$), alkoxyl($RO^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$) andnon radical species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$) andperoxynitrite ($ONOO^-$)[9,10]. Protein modifications caused by ROS/RNS include formation of carbonyls, dityrosine and nitrated and chlorinated tyrosine. Oxidative stress induced peroxidation of membrane lipids can lead to alterations in the biological properties of the membrane, such as the degree of fluidity and inactivation of membrane

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bound receptors or enzymes, which in turn may impair normal cellular function and increase tissue permeability[11]. Not all reactive species ($O_2^{\bullet-}$ and H_2O_2) react with DNA bases or with the deoxyribose. However, the most important oxygen-free radical causing damage not only to DNA but also to lipids and protein is the hydroxyl radical ($HO^{\bullet-}$). Lipid peroxidation products including the aldehydes also react with DNA, phospholipids and proteins, to generate stable products that are thought to contribute to the pathogenesis of numerous diseases. Chemical or immunochemical quantification of these relatively stable products as a biomarker of OS is available and numerous numbers of these markers have been studied in disease conditions.ROS/RNS are generally too reactive and/or have a short half-life period. However, molecular products formed from the reaction of ROS/RNS with biomolecules instead are generally considered more stable than ROS/RNS themselves[11-14]. Chen S (2012) measured MDA and Antioxidant enzyme activities to understand the relationship between Oxidative stress in Metabolic Syndrome[15]. Similarly, a LIPGENE study (2013) analyzed Lipid peroxidation product and Hydrogen peroxide to determine Oxidative Stress in metabolic syndrome[16] while Butkowski*et al.* (2016) investigated Glutathione and 8-hydroxy-2-deoxyguanosine in chronic hyperglycemia[17]. The literature is very heterogeneous and does not report on the relative oxidative damages caused to DNA, lipid and protein. Added to the above and the non-existent data from distant outreaches of the nation, we propose to study the redox status of individuals by measuring the concentrations of these stable oxidation target products in those with MetS and those progressing towards MetS. We have emphasized on 8-OH-deoxyguanosine, as a marker of DNA oxidative damage, malondialdehyde (MDA), hydroxynonenal (HNE) as a marker of lipid oxidative damage and protein carbonyl (PC), advanced oxidation protein product (AOPP) as a marker of protein oxidative damage including total antioxidant capacity. We investigated if the oxidation markers of DNA, lipid and protein increased exponentially with an increase in the risk parameters of MetS.

Materials and Methods

The study was a hospital-based cross-sectional design that was conducted in the Department of Biochemistry, Central Referral Hospital, Sikkim Manipal Institute of Medical Sciences. Sample selection and collection were performed in the hospital's phlebotomy lab after obtaining due permission from the Institutional Ethics Committee [IEC/192/12-05(a)]. Outpatients (also including those who came for annual health checkups between March 2014-December 2016 with a requisition for biochemical investigations like fasting blood sugar and lipid profile were enrolled for the study. Patients were informed of the study and those willing were asked for a signed consent. General information on age, sex, anthropometric measurements, ethnicity, smoking/alcohol habits, present medications and history of past and present diseases were recorded.

Blood pressure and waist circumference of all participants were measured by standardized procedures. Blood pressure was recorded by an auscultatory method using sphygmomanometer. After the patient was comfortably seated an average of two readings was taken at an interval of 2 min. Waist circumference was measured using a non-stretchable tape at the umbilical scar level in between lowest rib and iliac crest.

A 3 mL fasting blood sample of all participants were drawn by the hospital phlebotomists and was used to measure fasting blood sugar and lipid profile using ERBA Kits for an ERBA Manheim EM 200 full auto analyzer. The blood sample was used for estimating DNA oxidation marker: 8-hydroxy-deoxy-Guanine (8-OHdG). 8-OH-dGwas assayed by EIA kit from CAYMAN chemicals USA. The assay is based on the competition between oxidatively damaged guanine species and 8-OH-dG-acetylcholinesterase conjugate (DNA/RNA oxidative damage tracer) for a limited amount of DNA/RNA Oxidative Damage Monoclonal Antibody. Because the amount of tracer is held constant while the concentration of oxidatively damaged guanine varies, the amount of tracer that is able to bind to the monoclonal antibody will be inversely proportional to the concentration of the oxidatively damaged guanine in the well.

Lipid peroxidation marker Hydroxynonenal was assayed by EIA kit from CELL BIOLABS, INCUSA. HNE in the sample binds to HNE conjugate pre-absorbed on the ELISA plate. An anti-HNE polyclonal antibody is added after incubation, followed by an HRP conjugated secondary antibody which was then determined by comparison with a predetermined HNE-BSA (μg/mL) standard curve at 450 nm; malondialdehyde was assayed by colorimetric kit from CAYMAN chemicals, USA. According to the procedure, the TBA adduct formed by the reaction of

MDA and TBA under high temperature (90-100°C) and acidic condition was measured colorimetrically at 530-540 nm expressed in μM concentration.

Protein oxidation marker: Advanced Oxidation Protein Product was assayed by a method described by Witko-Sarsat V *et al.*[18]. The unknown AOPP-containing samples or Chloramine standards were mixed with Potassium Iodide (an initiator) that begins a color development process. After a brief incubation, a stop solution Glacial Acetic Acid was added and the samples and Chloramine standards were read with a standard spectrophotometer.

Protein carbonyl on the other hand was determined by Buss IH[19]. The protein carbonyl colorimetric assay utilizes the DNPH reaction to measure the protein carbonylcontent in samples. The amount of protein-hydrazone produced is quantified spectrophotometrically at an absorbance of 375 nm in a Lab Life ER 2007, microplate reader.

Study group

Participants (male and female ≥ 20 yrs of age) were randomly evaluated for MetS risk parameters following Harmonized IDF definition[20]. MetS is diagnosed when the patient has three or more of the following five risk parameters; Fasting glucose ≥ 100 mg/dL (or receiving drug therapy for hyperglycemia), Blood pressure ≥ 130/85 mmHg (or receiving drug therapy for hypertension), Triglycerides ≥ 150 mg/dL (or receiving drug therapy for hypertriglyceridemia), HDL-C <40 mg/dL in men or < 50 mg/dL in women (or receiving drug therapy for reduced HDL-C).Waist circumference is ethnic specific: ≥ 90 cm in men or ≥ 80 cm in women for Asian Indians]. Based on the subject selection criteria and presence of the number of risk factors of MetS, participants were subdivided into 4 study groups (Table 1).

a) 3 Risk/MetS: were MetS diagnosed subjects that had 3 or more risks factors, for example, large waist circumference + elevated blood sugar + elevated blood pressure.

b) 2 Risk: were those having 2 Risk factors, for example, elevated blood pressure + raised fasting blood sugar.

c) 1 Risk: were those having 1 Risk factor for example only a large waist circumference.

d) 0 Risk: were those having 0 Risk factor.

The subject selection criteria

Inclusion criteria: Participants ≥ 20 years of age and anyone not under long-term medication for any diseases other than for Diabetes, Hypertension, Dyslipidemia were included. Exclusion criteria: Pregnant ladies, smokers and alcohol users were excluded.

Sample size

Sample size for pair wise comparison was calculated according to Wang H *et al.*[21]. Assuming 5% level of significance (α= 0.05), power 80% (β= 0.84); mean difference (δ) of 67.5 and standard deviation (σ) of 60.2, a sample size (n) =12 was found in each group;

$$n= [2\ (Z_\alpha + Z_\beta)^2\ \sigma^2/\delta^2].$$

However, we considered 40 participants under each group (Group 1; 3 Risk /MetS =40, Group 2; 2 Risk= 40, Group 3; 1 Risk =40 and Group4; 0 Risk =40).

Statistical analysis

All Statistical analysis was performed using SPSS 20 software package (IBM SPSS Statistics).Data were inspected for normal distribution and accordingly appropriate tests were adopted A Kruskal-Wallis test was conducted to determine significant differences in

Table 1 — Metabolic syndrome risk parameters in the four study groups

	Metabolic syndrome (n=40)	Non metabolic syndrome (n=120)		
	Subjects with 3 or more Risk for MetS (n=40)	Subjects with 2 Risk for MetS (n=40)	Subjects with 1 Risk for MetS (n=40)	Subjects with 0 Risk for MetS/Control group (n=40)
Age (in years)	47 ± 11	45 ± 13	39 ±10	33 ±9
Waist Circumference (cm)	95.5 ± 8.2	95.1 ± 8.6	88.5 ± 9.3	82.3 ± 7.0
Systolic blood Pressure (mmHg)	130 ± 14	124 ± 10	120 ± 6	118 ± 6
Diastolic blood pressure (mmHg)	85 ± 7	83 ± 8	79 ± 4	77 ± 7
Fasting blood sugar (mg/dL)	131.7 ± 120	106 ±56	93 ± 11	86 ± 10
Triglyceride (mg/dL)	196.1 ± 120	139.9 ± 59	158.6 ± 157	96.9 ± 16
HDL-C (mg/dL)	50.4 ± 18	51.8 ± 15	61.7 ± 24	54.9 ± 16

Values are mean± SD

the oxidation products of Lipid, Protein, DNA and Total Antioxidant Capacity across groups with "3 Risk/MetS, 2 Risk, 1 Risk and 0 Risk" for MetS. A P value of < 0.05 was taken statistically significant. Pair wise comparisons of the oxidation markers were performed for those significant in the Kruskal-Wallis test using Dunn's procedure with a Bonferroni correction for multiple comparisons and for this statistical significance was accepted at P <0.012. Values were median scores unless otherwise stated.

Results

PC and AOPP, as a marker of protein oxidative damage, differed significantly across the four study groups at P =0.009 and 0.013, respectively, (Fig. 1A). MDA & HNE as a marker of lipid peroxidation did not differ significantly across the four study group at P =0.608 and 0.456, respectively, (Fig. 1B). However, DNA oxidative damage product "8-OH dG" differed significantly at P =0.007 (Fig. 1C). TAC as well did not show significant difference across the group at P =0.199 (Fig. 1D). Subsequently, to discover which group is different to which other group, a pair wise comparison were performed for statistically significant protein oxidative and DNA oxidative damage markers. This post hoc analysis revealed statistically significant differences; in 8-OH dG between 0 Risk-3 Risk, at P =0.005 but not between 0 Risk-2 Risk and 0 Risk-1 Risk (Table 2). Likewise, PC and AOPP did not differ significantly between 0 Risk -3 Risk, 0 Risk -2 Risk and 0 Risk -1 Risk (Table 2).

Discussion

We tested stable but indirect markers of oxidative damage products of macromolecules including MDA and Hydroxynonenalas lipid peroxidation product; Advanced Oxidation Protein Product and Protein Carbonyl as protein oxidation marker and 8-OHdG as DNA oxidation marker and examined their status across the four groups with 3 Risk, 2 Risk, 1 Risk and 0 Risk for MetS, so as to determine if the level of oxidative damage markers increased with an increase in the number of risk factors of MetS. Upon careful examination, we observed "oxidation markers of DNA, lipid and protein did not increase with an increase in the risk parameters of MetS". The lipid per oxidation marker Malondialdehyde and 4-Hydroxynonenal, the two most widely used markers for lipid peroxidation and oxidative stress *in vivo* unfortunately did not demonstrate statistically significant difference on clustering of the risk factors, represented by those having 3 Risk, 2 Risk, 1 Risk and 0 Risk for MetS.
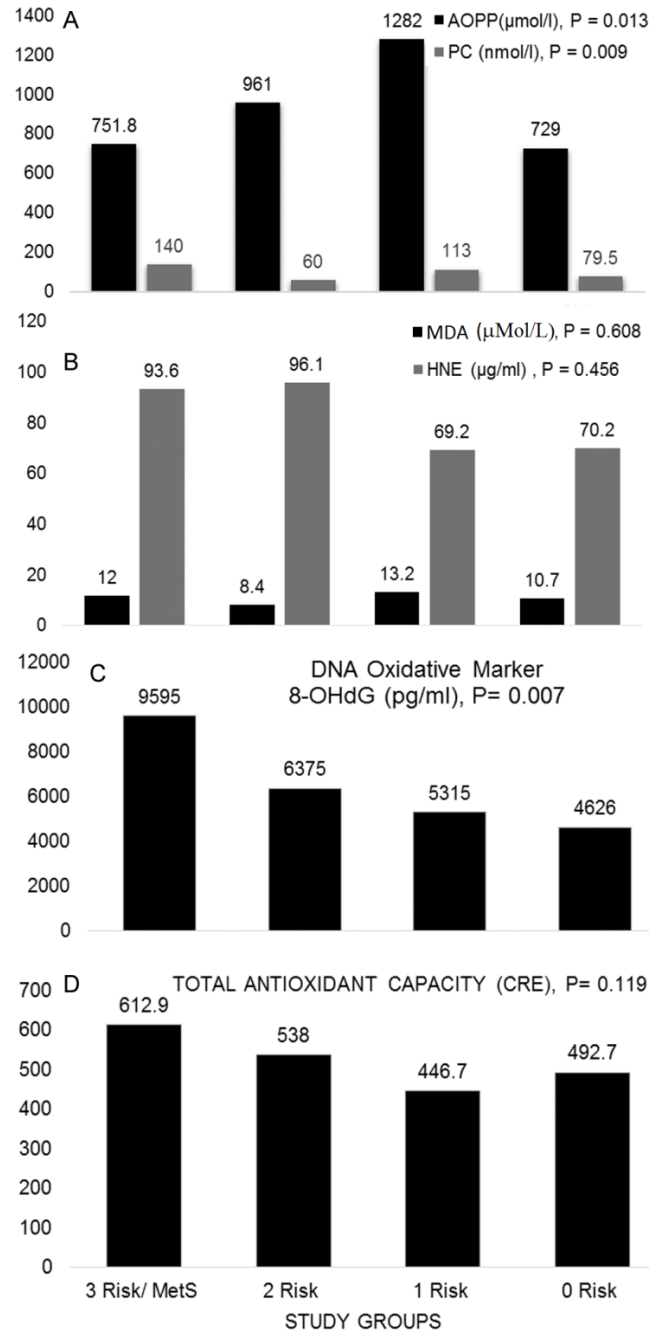

Fig. 1 — (A) Comparison of Advanced Oxidation Protein Product (AOPP) and Protein carbonyl (PC) in 3 Risk, 2 Risk, 1 Risk and 0 Risk of MetS by Kruskal -Wallis *H*-test (significant at P <0.05). AOPP; statistically significant, P =0.013*; and PC (nM/mL); statistically significant, P =0.009*; (B) Comparison of Malondialdehyde (MDA) and Hydroxynonenal (HNE). MDA (μM/L); statistically insignificant, P =0.608; and HNE (μg/mL); statistically insignificant, P =0.456; (C) Comparison of DNA Oxidation product; 8-OHdG (pg/mL) across groups with statistically significant, P =0.007*; and (D) Comparison of TAC (CRE); statistically insignificant, P =0.119

Table 2 — Post-hoc analysis for multiple comparisons between 0-1, 0-2, 0-3 Risk for PC, AOPP and 8-OHdG (Adjusted *P*-values are presented)

	Significance
Protein carbonyl	
0 Risk – 1 Risk	0.276
0 Risk – 2 Risk	1.000
0 Risk – 3 Risk	0.035
Advanced Oxidation Protein Product	
0 Risk – 1 Risk	0.012
0 Risk – 2 Risk	0.404
0 Risk – 3 Risk	1.000
8-hydroxy, 2-deoxy guanosine	
0 Risk – 1 Risk	0.416
0 Risk – 2 Risk	1.000
0 Risk – 3 Risk	0.005*

*Significant at $P<0.012$

Oxidative stress also increases protein oxidation[22]. Protein carbonyl is generated on direct oxidative damage to the protein backbone, specifically to amino acids such as Lysine, Arginine, Histidine, Proline, Glutamic acid and Threonine, or by binding of aldehyde produced from lipid peroxidation such as 4-HNE or acrolein[22,23]. AOPP on the other hand can be generated by chlorinated oxidants such as chloramines or hypochlorous acid[18] and neutrophills which constitute the most important source of chlorinated oxidants due to their high content in myeloperoxidase is reported to be involved in plasma AOPP formation[24] but once again AOPP and PC as a marker of protein oxidation in MetS did not demonstrate significant differences between those with the syndrome and those without the syndrome. Nevertheless, it was noteworthy that although statistically insignificant the level of protein and lipid peroxidation markers were higher in the 3 Risk, 2 Risk and 1 Risk group compared to the 0 Risk group.

Biomarkers of Oxidative Stress Study (BOSS), using acute CCl4 poisoning in rodents as a model for OS, has demonstrated that 8OHdG in urine is a potential candidate as a general biomarker of oxidative stress[25] of the markers tested for significant differences across the study group, only DNA damage marker, 8-OHdG demonstrated significant statistical difference in the study population despite their having been on blood pressure and glucose-lowering medications. This difference was found between 3 Risk-0 Risk group. In other words, 8-OHdG level was higher in those categorized MetS (3 Risk group) when compared to those categorized control (0 Risk). This oxidized guanine species is the by-product released during the repair of the oxidative damage to guanine on both DNA and RNA. Furthermore, guanosine is the most oxidized among the DNA nucleobases[26]. Its level was reported to increase in hypertension[27], cancer, cardiovascular disease[26-29] and diabetes[30,31]. There is indeed a cumulative and synergistic effect of the risk factors of MetS, clustering of 3 and more risks factors of MetS has a greater effect on DNA damage than those with 0 Risk possibly due to tremendous OS[32] put forth by the risk factors (Fig. 1C). Presence of either a 2 Risk or 1 Risk increased OS erratically. If presence of even a single risk factor increased OS as much as those with 2 Risk was unclear from our current experiment. Lee J *et al.* demonstrated oxidative DNA damage as illustrated by 8-OHdG occurred more in their hypertensive patients[27].Gursatej Gandhi *et al.* also reports significant DNA damage differences in their hypertensive and normotensive patients[33]. All of this implies hypertension associated with oxidative stress is the reason for the genomic instability. Low levels of HDL cholesterol increase the risk of heart disease. And finally, abdominal obesity measured using a simple non-stretchable tape at the umbilical scar level is known to increase DNA damage by multiple mechanisms. Obese people often have chronic low-level inflammation, which can, over time, cause DNA damage. Secondly, adipose tissue produces excess amounts of estrogen and inflammatory cytokines which have been associated with increased risks of cancers[34]. Infact all the 5 Risk parameters of MetS contributes towards OS and consequently induces DNA damage. Further, estimation of Total Antioxidant Capacity (TAC), that was performed to understand the overall redox status in the study participants, did not differ significantly across the groups that had 3 Risk, 2 Risk, 1 Risk and 0 Risk for MetS. Our findings illustrated a completely opposite TAC situation, wherein instead of participants under the 0 Risk category, participants those in 3 Risk, 2 Risk and 1 Risk category had a higher TAC status (Fig. 1D). Mates JM and colleagues proposed when OS arises as a consequence of a pathologic event, a defense system promotes the regulation and expression of antioxidant enzymes[35]. Aouacheri O *et al.* reported SOD activity increased markedly in Type 2 Diabetic patients when compared to healthy subjects[36]. Another study conducted by Gupta S *et al.* showed that in the early stages of CAD, Superoxide Dismutase and Catalase levels increased to protect and prevent lipid peroxidation whereas they decreased significantly with the worsening of the

disease[37]. Hence if the antioxidant enzymes can rise in response to early OS so should the non enzymatic antioxidant. TAC assays in the plasma are a measure of low molecular weight, chain-breaking antioxidants that excludes the contribution of antioxidant enzymes and metal binding proteins.

Conclusion

Oxidative stress markers of DNA, lipid and protein do not increase with an increase in the risk parameters of MetS. However, it is indeed high in those diagnosed with MetS with 3 and more risks, compared to those with 0 Risk. Presence of 2 or 1 Risk also increases OS compared to 0 Risk. There is oxidative stress damage in MetS to lipid and protein but DNA damage was of significant consequence. The nucleic acid is not only targeted by the direct markers of OS such as hydroxyls radical but the oxidation products obtained upon oxidative damage to proteins and lipid induces additional harm to the nuleic acid to increase the damage load.

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