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COORDINATORE Professor Persio Dello Sbarba

A molecular epidemiology study on MDA adducts in a representative sample of the general population and in cancer patients

Settore Scientifico Disciplinare MED/04

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Introduction

Many studies in the field of chemical carcinogenesis and molecular epidemiology have focused on the analysis of DNA adducts induced from exposures to environmental carcinogens. Epidemiological data show that there are lots of endogenous metabolic products with the same carcinogenetic properties, even if they are not yet studied as well (Burcham 1998).

Malondialdehyde (MDA) is one of these; it is a highly reactive three carbon dialdehyde chemical compound. Under physiological condition (ph 7,4), MDA exists as an enolate anion, a form that is only fairly reactive, forming Schiff bases with molecules containing a free amine group. Instead beta-idroxyacrolein is the predominant form under more acid condition (ph<4) and it is a very reactive electrophile, able to react in a Michael addition with a number of biologically important nucleophiles.
According to the literature, basal plasma MDA levels are between 0,3-38 μM/L (Badcock N 1997 , Lee 1980, Yeo 1994, Wasowics 1993), while basal MDA serum levels are between 0,9-47 μM/L) (Suematsu 1977).

MDA is metabolized in the liver to malonic acid and semialdehyde. This is unstable and spontaneously decomposes to acetaldehyde that is then converted to acetate by aldehyde deydrogenase and finally to carbon dioxide and water. Some MDA finally ends up as acetyl-CoA. Mammalian urine also contains enaminals derived from hydrolysis of MDA modified proteins (Esterbauer 1991). The urinary output of MDA in human is typically 0,2-0,8 μM/L (Tomita 1990).

MDA can react with aminoacids, proteins, DNA forming a variety of adducts and crosslinks. When it reacts with DNA bases, it produces a variety of mutagenic compounds. Furthermore, MDA has the potential to induce amino-imino-propen cross-links between complimentary strands of DNA and can also produce the formation of DNA-protein cross-links (Esterbauer H, 1991; Badcock, N, 1997; Yeo H C, 1994)

Background

MDA biochemical formation

MDA is a naturally occurring product of lipid peroxidation and prostaglandine biosyntesis.

The start up for lipid peroxidation is the presence of ROS (radical oxigene species). ROS can occur both because of outside agents (chemical, biological, physical ones) or inside organism inflammation (in this second scenery polymorphonuclear cells, macrophages are involved). The major target of lipid peroxidation is the cell membrane.
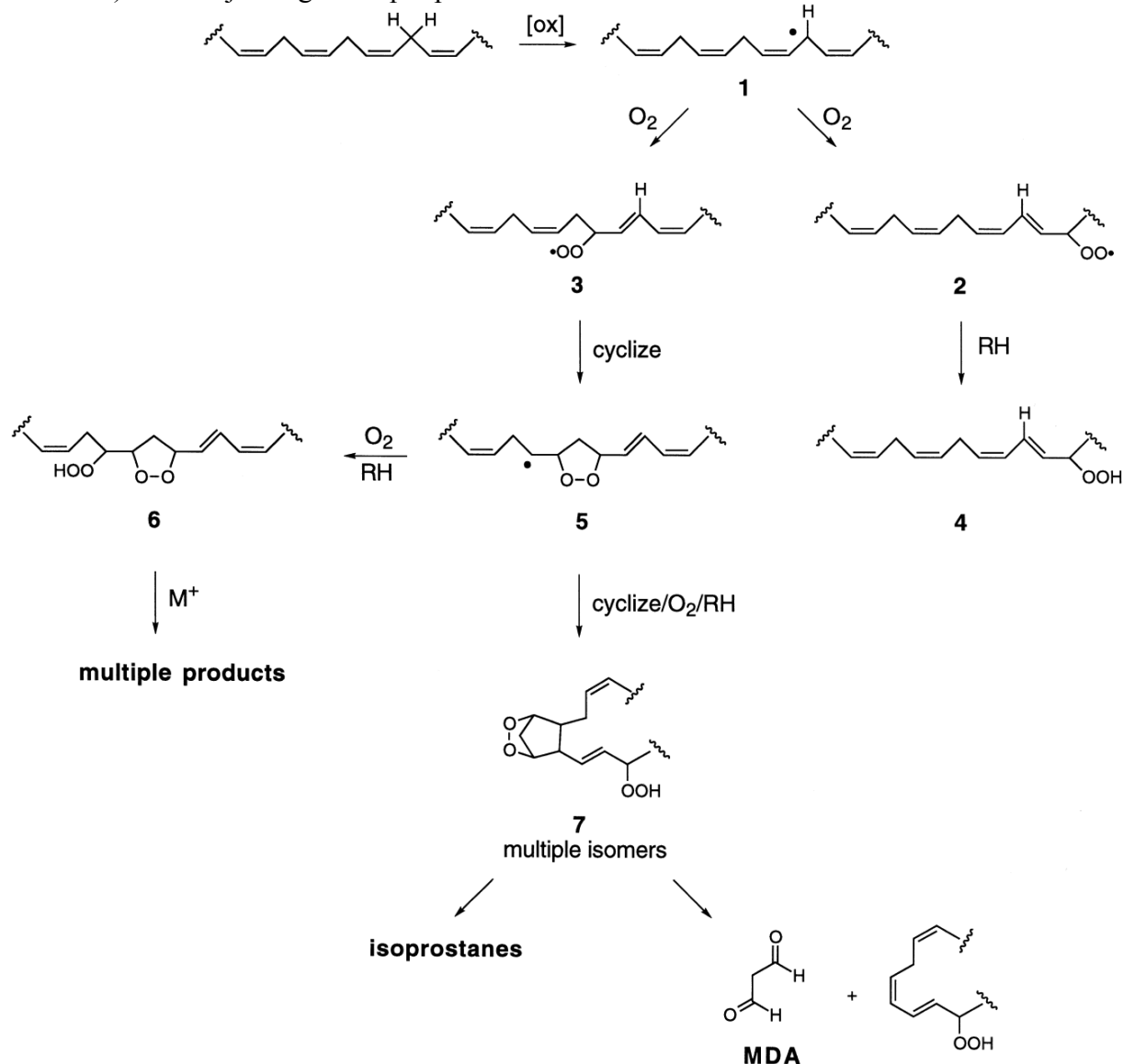

Figure 1. Lipoperoxidation pathway (from Marnett 1999).

Polyunsaturated fatty acids have inside one or more methylene groups located between cis double bonds. The methylene groups are very reactive to oxidizing agents and their

hydrogen atoms are removed to form carbon-centered radicals (molecule 1). Carbon-centered radicals react with oxygen molecule forming peroxyl radicals at a rate limited only by diffusion. Therefore, the initial products of polyunsurared fatty acid oxidation are peroxyl radicals (molecules 2, 3). The fate of the peroxyl radical depends on its position in the carbon chain of the fatty acid.

When the peroxyl radical is present at one of the two ends of the double bond system, it is reduced to a hydroperoxide. Conjugated diene hydroperoxides are the simplest products of this reaction and they represent the end lipid peroxidation products in the absence of metals (molecule 4). In phospholipid membranes, the molecules that reduce peroxyl radicals to hydroperoxides are either another molecule of fatty acid or vitamin E.
When another molecule of fatty acid reduces the peroxyl radical, a new carbon-centered radical is generated propagating the fatty acid oxidation. Through this radical chain process, one oxidizing agent can oxidize many molecules of fatty acid.

If the peroxyl radical is at an internal position in the fatty acid chain (molecule 3) the most frequent reaction is the cyclization to an adjacent double bond instead of the immediate reduction to a hydroperoxide,
The cyclization product (molecule 5) is a cyclic peroxide adjacent to a carbon-centered radical. This carbon-centered radical has two fates. It can couple with an oxygen molecule in order to form a peroxyl radical which is reduced to a hydroperoxide (molecule 6) in the same way as described previously for molecules 2-4.; otherwise, the carbon-centered radical (molecule 5) can undergo a second cyclization to form a bicyclic peroxide, which after coupling to an oxygen molecule and reduction, yields a molecule (molecule 7) structurally analogous to the prostaglandin endoperoxide, PGG2. Compound 7 is a common intermediate for the production of isoprostanes and malondialdehyde MDA, through chemical conversion of the bicyclic peroxide group. The fragmentation that produces MDA generates a 17-carbon fatty acid as the other product (Marnett 1999).

Thus, one oxidizing agent can cause many molecules of fatty acid to become oxidized, through radical chain process. The actual lengths of the radical reactions that happen in cells are sensitive to various elements but in pure chemical systems approximately 60 molecules of linoleic acid and 200 molecules of arachidonic acid are consumed per initiation event. The most determinant of the length of radical chains in vivo is represented by the concentration of vitamin E that can be found in the phospholipids bilayer. Vitamin E reduces peroxyl radicals that generally breaks the radical chain and slows down the percentage of lipid peroxidation. However, under conditions of very low level of oxidant, vitamin E has actually been demonstrated to initiate another radical chain. (Marnett 1999)

MDA is not the only lipid peroxidation product. As we have already seen, monohydroperoxides are always the primary products of lipid peroxidation. usually mentioned as lipid hydroperoxides (LOOHs). The number of positional isomeric monohydroperoxides that can be formed from a given PUFA depends on the number of double bonds (n) and is equal to 2n-2. For example, two monohydroperoxides, with the hydroperoxy groups either at carbon atom 9 or carbon atom 13 (9-OOH, l3-OOH), come from peroxidation of linoleic acid (18:2). Linolenic acid (18:3) yields four hydroperoxides, arachidonic acid (20:4) six, eicosapentaenoic acid (20:5) eight, and docosahexaenoic acid (20:6) yields 10 different monohydroperoxides. Upon the existing condition (for example referring to temperature, metals ions traces, ph, other components) hydroperoxides give second and tertiary reactions, that leads to the formation of other molecules.

These other lipid peroxidation products may be roughly split into three categories:

1) Chain-cleavage products such as: n-alkanals, 2-alkanals, 2,4-alkadienals, alkatrienals, hydroxy-aldehydes, 4-hydroxyalkenals, malonaldehyde, alcohols, ketones, furanes, lactones, alkanes and alkenes. Most of these substances result from the breakup of the two C-C bonds adjacent in the hydroperoxy group by the so called β-cleavage reaction. A certain PUFA will always give the same pattern of methyl fragments. Most of the oxidation products belong to this class of methyl-terminus-cleavage products.

2) Products formed by rearrangement of the monohydroperoxides or rearrangement and consecutive oxidation. This involves five-membered monocyclic peroxides, hydroperoxi-epidioxides, dihydroperoxides, bicyclic-endoperoxides, and monohydroxy, dihydroxy, trihydroxy, ketohydroxy and epoxihydroxy compounds.

3) Higher-molecular-weight oxidation products resulting from di- and polymerization reactions leading to intermolecular ether-, peroxy-, and C-C cross links between the peroxidised lipid molecules (Esterbauer 1993).

The following pictures show the second way MDA can be formed; pictures refer to the prostaglandins biosynthesis pathway.
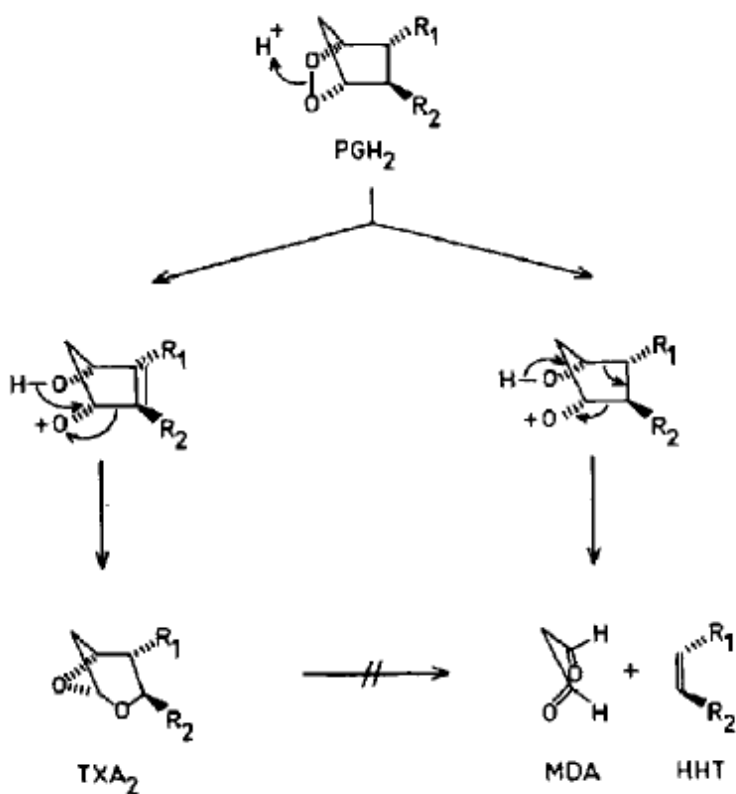
Diczfalusy 1977
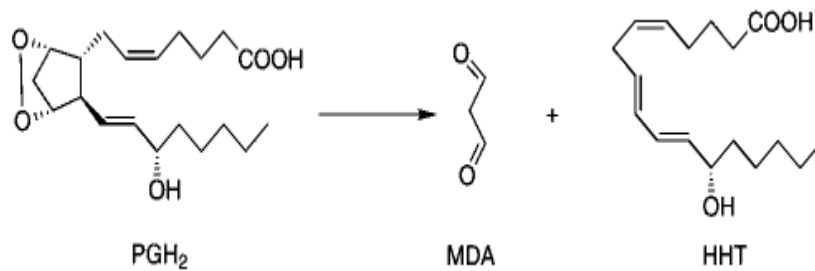
Figure 2. *Prostaglandins pathway: MDA generation.*

MDA can be formed during cyclooxygenase (COX) catalysis in platelets. In vitro studies strongly suggest that platelet thromboxane syntase can lead to the production of C17 hydroxy acids and malondialdehyde.

The reaction from prostaglandin H2 (PGH 2) to tromboxane, mediated by tromboxane syntase, is based on the protonation of the oxygen on C9 and it is an isomeration reaction. The cation obtained from the cleavege of the O-O bond can rearrange in an unstable molecule, that convert itself spontaneously to MDA plus C17 hydroxyacid (Diczfalusy 1977). Otherwise this reaction can be catalyzed by ferrous ion or heme (Plastaras 2000). Some studies suggest that this reaction produces tromboxane A2, hydroxyheptadecatrienoic acid, and MDA in a 1:1:1 ratio (Plastaras2000). Plastaras and collegues found that the breakdown of PGH2 to MDA and hydroxyheptadecatrienoic acid can occur also via liver microsomes (P450) .

MDA properties: genotoxicity, mutagenicity and carcinogeneticy.

MDA is genotoxic, mutagenic and carcinogenic, in a sort of logical chain of consequent steps. MDA reacts with nucleic acid bases to form multiple adducts. These adducts are shown in the next picture.
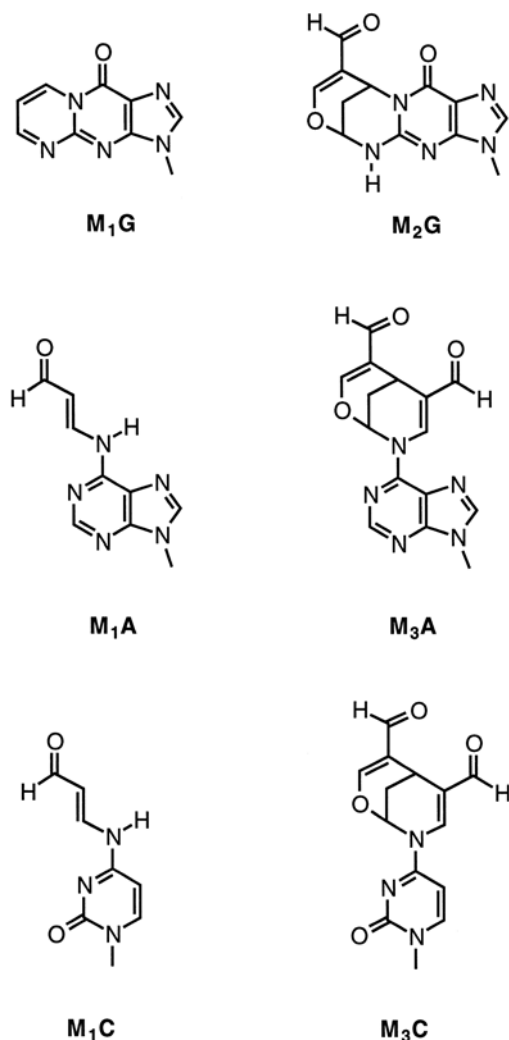
Figure 3. *Mda-DNA adducts (Marnett 1999)*

Both MDA carbonyl equivalent generate adducts to N2 and N1 of deoxyguanosine (dG), with loss of two water molecules to form a pyrimidopurinone. This compound is a planar cyclic and aromatic molecule that is fairly fluorescent (3% quantum yield).

The condensation compounds with deoxyadenosine (dA) and deoxycytidine (dC) come from the addition of one of the carbonyl equivalents of MDA to the exocyclic amino groups and form an oxopropenyl derivative. There is no evidence for cyclization of these products.

The comparison of the various adducts, that are produced in the reaction of MDA with DNA in vitro, indicates that the pyrimido (1,2α purin)-10(3*H*)-one (M_1dG) is the major adduct, followed by *N* 6-(3-oxo-propenyl)-deoxyadenosine (M1dA). The amount of M_1dG is approximately five times that of M1dA. *N*4-(3-oxopropenyl)deoxycytidine (M1dC) is formed in only trace amounts (Marnett 1999).

Considering the percentage production, the order of these adducts is M1dG>M1dA>M1dC, with the M1dG (detected in liver, pancreas, breast and leukocytes) representing around 1–4

per 108 nucleotides in healthy individuals., MDA-DNA adducts appear in much higher percentage at mitochondrial rather in nuclear DNA, probably for the absent of the nucleotide excision repair mechanism (the major mechanism responsible for the repair of the M1dG) in mitochondrion. (Voulgaridou 2011)

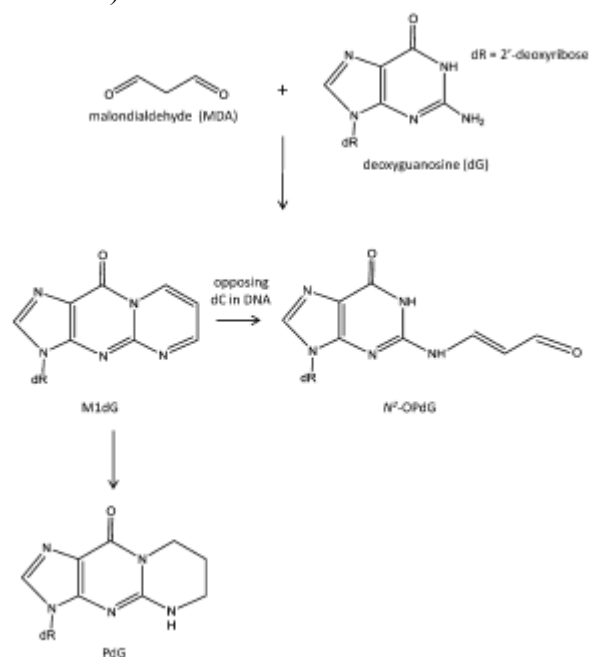*Figure 4.*M1dG has a close external ring configuration when it is opposite to a deoxythymidine (dT) or when it is on a single stranded DNA; instead the external ring opens through hydrolysis forming the noncyclic N2-(3-oxo-1-propenyl)-2-deoxyguanosine (N2-OPdG) when it is opposite to a dC (Volgaridou 2011).

The polymerization of MDA in dimers and trimers that also react with DNA is a complicating element of the reaction of MDA with nucleosides.
The dimer of MDA reacts with dG to form an oxadiazabi-cyclo(3:3:1)nonene derivative, whereas the trimer of MDA reacts with dA and dC to form (5',7'-bis-formyl-2'*H*-3',6'-dihydro-2',6'-methano-1',3'-oxazocin-3') derivatives of the exocyclic amino groups. The oligomerization of MDA is relatively slow at neutral pH so the monomeric adducts described previously are the major products generated under physiological conditions. However, there may be conditions in vitro or possibly in vivo where these unusual multimeric adducts are formed. The ability of MDA to give monomeric and oligomeric adducts come from the nucleophilic reactivity of its enol functional group whereas the electrophilic reactivity is a reflection of its aldehyde functional group. (Marnett 1999)

Dedon et al. demonstrated that M1dG, and presumably other MDA-derived adducts, can be produced independently of lipid peroxidation and of prostaglandine generation pathway. (Marnett 1999). The direct oxidation of each carbon in deoxyribose lead to a unique spectrum of sugar residues or oxidized abasic sites. Among these products there are several reactive electrophiles capable of forming mutagenic adducts with DNA bases. (Xifeng Zhou 2006)
Direct oxidation of DNA by agents that abstract the 4'hydrogen atom of the sugar backbone (for example, calicheamicin, bleomycin) starts a cascade of reactions that yields to the formation of base propenals.

Base propenals are oxopropenyl base derivatives, structurally similar to acroleins, substituted at the β position with good-to-moderate leaving groups. These compounds (including M1dA and M1dC) transfer their oxopropenyl group to dG to form M1dG. Treatment of DNA with bleomycin or calicheamicin, in the complete absence of lipid, leads to the formation of M1dG.

M1dG also is formed by the direct reaction of adenine propenal with DNA. This is another pathway linking oxidative stress to the formation of M1dG in DNA and may explain the occurrence of M1dG at high levels in certain human tissues.(Marnett 1999). Also other Authors have described in detail how this chemical reaction moves. (Awada 2001, Pratviel 1995, Rashid 1999, Henner 1983)
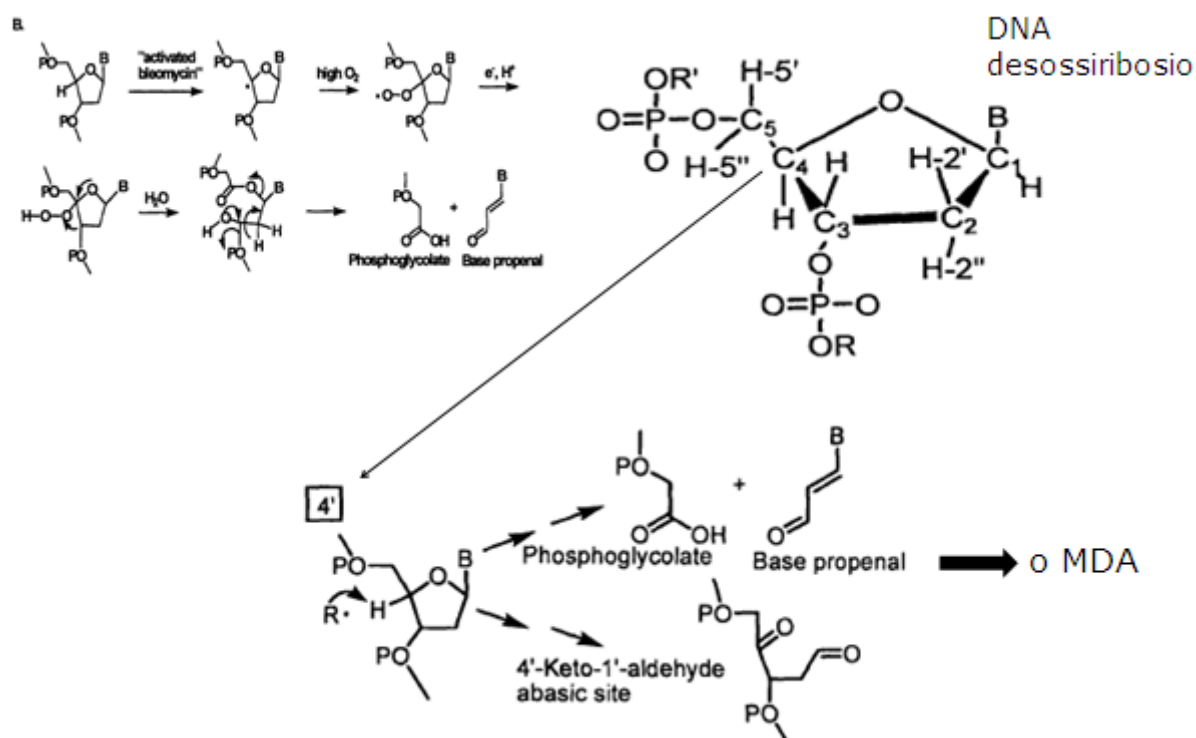
Figure 5. *Direct oxidation of DNA and MDA-DNA adducts. (partly rearranged from Xifeng Zhou 2006)*

Mukai and Goldstein showed the mutagenic activity of MDA toward *Salmonella typhimurium* in 1976. Because of MDA unstability, the material used for the assay was prepared by hydrolysis of tetraethoxypropane.

Marnet and Turtle (1980, experiment on *Salmonella typhimurium*) demonstrated that some impurities, formed during tetraethoxypropane hydrolysis, are nearly 30-fold more mutagenic than MDA and represent a significant part of the activity detected in the assay (roughly 50%). However, Basu and Marnett (1983) verified in a latter experiment (on Salmonella typhimurium) that the assay of highly purified MDA, prepared by three independent means, is mutagenic itself (Marnett 1999).

MDA induced genotoxicity has also been demonstrated in several mammalian systems, including mutagenicity in murine L5178Y lymphoma cells (Yau, 1979); chromosome fragmentation and micronuclei formation in rat dermal fibroblasts (Bird et al., 1982); single-strand breaks and sister chromatid exchanges in CHO (chinese hamster ovary) cells (Brambilla et al., 1985), (Burcham 1998).

The mutagenic potential of MDA–DNA adducts, has been analysed using random and site-specific approaches. Benamira et al. reacted MDA, at neutral pH, with a single-stranded M13 vector containing the *lacZ*a gene and scored mutations to the *lacZ*a- phenotype, after replication in strains of *Escherichia coli* induced for the SOS response (SOS response is a global answer to DNA damage in which cell cycle is arrested and DNA repair and mutagenesis are induced). Increasing concentrations of MDA, led to an increase in *lacZ*a-mutations, coincident with the growth in the level of the major MDA-deoxyguanosine adduct, M1dG.

The most common sequence changes induced by MDA, were base pair substitutions (76%). About 43% (29/68) were transversions, most of which were G to T (24/29). Transitions counted for 57% of the base pair substitutions (39/68) and were comprised exclusively of C to T (22/39) and A to G (17/39). Frameshift mutations were identified in 16% of the induced mutants and consisted of mainly single base additions occurring in runs of repeated bases (11/14). Assuming all mutations are targeted to the site of adduction, the property of MDA to induce base pair substitution mutations at dG, dA, and dC residues, corresponds with its capability to make adducts at all three deoxynucleosides. No mutations were detected at dT residues as expected from the fact that MDA does not form detectable adducts to dT. (Marnett 1999)

Vanderveen and collegues found that MDA induces frameshift mutation and base pair substitution in bacteria and in mammalian cells. The Authors used site-specifically modified single- and double-stranded vectors to analyse the mutagenic potential of M1dG in bacteria and mammalian cells. M1dG induced frameshift mutations when placed in a reiterated (CpG)4 sequence but not when positioned in a nonreiterated sequence in *Escherichia coli* and in COS-7 cells. The frequency of frameshift mutations was greatest when M1dG was positioned at the third G in the sequence. M1dG induced base pair substitutions at comparable frequencies in both sequence contexts in COS-7 cells.(Vanderveen 2003)

Niedernhofer and collegues found evidence that MDA induces DNA interstrand cross-links. The Authors reacted MDA with pSP189 shuttle vector DNA, to evaluate the mutagenic potential of MDA in human cells; then they transfected them into human fibroblasts for replication. MDA induces up to a 15-fold increase in mutation frequency in the *supF* reporter gene compared with untreated DNA. Sequence analysis revealed that the majority of MDA-induced mutations occurred at GC base pairs. The most frequent mutations were large insertions and deletions, but base pair substitutions were also detected. MDA induced mutations were completely abolished when the adducted shuttle vector was replicated in cells lacking nucleotide excision repair. MDA induction of large deletions and the apparent requirement for nucleotide excision repair implies the possible involvement of a DNA interstrand cross-link as a premutagenic lesion. Indeed, MDA formed interstrand cross-links in duplex plasmids and oligonucleotides. Substrates containing the sequence 5'-d(CG) were preferentially crosslinked, consistent with the observation of base pair substitutions in 5'-d(CG) sites in the MDA induced mutation spectrum (Niedernhofer 2003)

Voitkun demonstrated that MDA could be one of the significant sources of endogenous DNA-protein crosslinks; the formation of DNA–protein crosslinks is restricted to proteins that are able to bind to DNA and in particular MDA yields to the creation of solid crosslink between DNA and histones under physiological ionic and ph conditions Crosslinking of histones to DNA proceeds through the initial formation of protein adduct followed by reaction with DNA. Modification of DNA by malondialdehyde does not lead to a subsequent crosslinking of proteins. (Voitkun 1999)

The spectrum of mutations caused by the naturally occurring DNA adduct Md1G, was defined also by site-specific analysis using M13 vectors replicated in *Escherichia coli* as shown in the experiments of Fink and colleagues. They discovered that M1dG, which is present endogenously in DNA of healthy individuals, is a strong block to replication and an efficient premutagenic lesion. M1dG was placed at position 6256 in the (-)-strand of M13MB102 by ligating the oligodeoxynucleotide 5'-GGT(M1G)TCCG-3' into a gapped-duplex derivative of the vector.

Unmodified and M1dG-modified genomes, including either a cytosine or thymine at position 6256 of the (+)-strand, were transformed into repair-proficient and repair-deficient *E. coli* strains, and base pair substitutions were quantitated by hybridization analysis. Modified genomes containing a cytosine opposite M1dG resulted in roughly equal numbers of M1dG to A and M1dG to T mutations, with few M1dG to C mutations. The total mutation rate was 1%, which represents a 500-fold growth in mutations, compared with unmodified M13MB102. Transformation of modified genomes containing a thymine opposite M1dG suggested an intrisical capability of M1dG to block replication. The (-)-strand was replicated >80% of the time in the unadducted genome but only 20% of the time when M1dG was present. Correction of the mutation frequency for the strand bias of replication showed that the actual frequency of mutations induced by M1dG was 18%. (Marnett 1999)

Experiments using *E. coli* with different genetic backgrounds suggested that the SOS response increases the mutagenicity of M1dG and that M1dG is a substrate for repair by the nucleotide excision repair complex (Fink 1997). The spectrum and frequency of mutations induced by M1dG were very close to those identified with a structural analog OPdG (*see previous picture from Volgaridou 2011 to have a look to both forms*) assayed in the same system.(Marnett 1999).

Anyway it can be assumed that both forms of MDA-induced DNA adducts are capable to induce mutations in mammalian cells, mainly G→A–T base pair substitutions as well as frame-shifts (with M1dG having a higher capability than OPdG). Anyway it is due to remember that such MDA lesions are mainly bypassed by the members of Y-family of DNA polymerases hPol and Dpo4 (homologue of hPol), which elongate DNA with low fidelity (Voulgaridou 2011)

Repair of M1dG was evaluated by transformation of M1dG-containing genomes into *E. coli* strains deficient in individual genes of DNA repair, based on the assumption that inactivation of a repair gene will enhance the half-life of the adduct and increase its mutagenicity in site-specific experiments. When cells were defective in formamidopyrimidine glycosylase or 3-methyladenine glycosylase, no effect on mutation frequency was detected. Instead those enzymes have been previously demonstrated to participate to the repair of other DNA adducts (8-oxodG and exocyclic adducts) (Marnett 2002).

About a fourfold increase in mutation frequency was detected when M1dG-containing genomes were transformed into cells deficient in nucleotide excision repair (either uvrA or uvrBy). A growth in mutation frequency of similar magnitude was observed also in OPdG-containing genomes. The capability of OPdG (and by analogy of M1dG) to be removed by reconstituted bacterial and mammalian nucleoside excision repair complexes was established by a series of in vitro experiments conducted by Jhonson, Fink and Marnett (Marnett 1999).
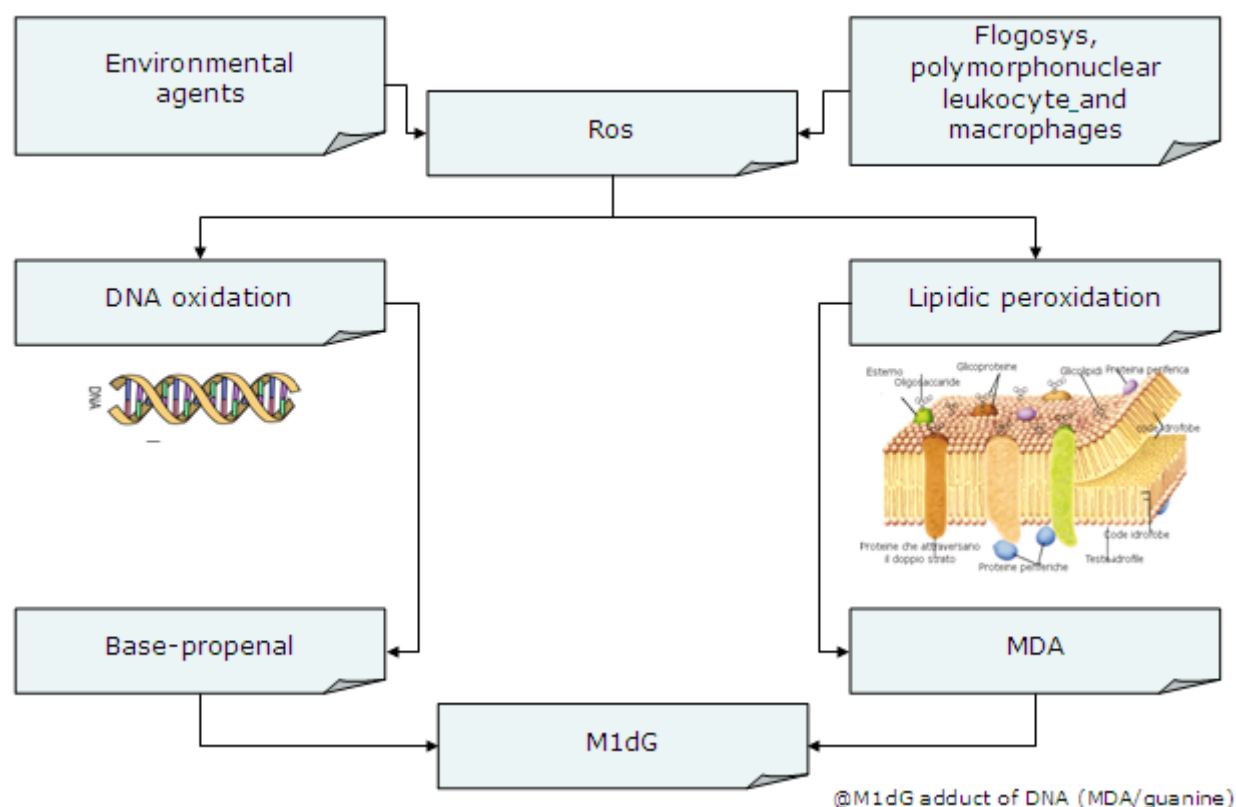
Figure 6. *This picture resumes what it has been illustrated in the text. ROS are the key points that lead to MDA formation. They can be induced from the interaction with external agents (chemical, physical, biological ones) or can be produced during inflammation processes. ROS can induce lipid peroxidation (which mainly happens in the contest of membrane cell, rich in phosoplipids and lipid acids), leading to MDA formation. MDA, interacting with DNA yields to the formation of Md1G adducts. Otherwise ROS, or substances that induce oxidation, can directly interact with DNA (C4 H abstraction), forming M1dG adducts.*

The production of MDA and its reaction with DNA to form mutagenic adducts constitutes a link between lipid peroxidation and clinical disease.

MDA-derived adducts are present in the genome of healthy humans at biologically significant levels. The levels reported are similar to those generated by exogenous chemicals in animal carcinogenesis experiments. MDA/DNA adducts are efficient premutagenic lesions that give mutations frequently observed in oncogenes or tumor suppressor genes from human tumors.

The major MDA-derived adduct is repaired by nucleotide excision repair. Moreover, DNA adduction by MDA correlates to alterations in cell cycle control and gene expression in cultured cells (Ji et al., 1998). So, lipid peroxidation must be recognized as a significant endogenous source of DNA damage and mutations that contribute to human genetic disease. (Marnett 1999*)

MDA was first identified to be carcinogenetic in 1972 by Shamberger and collegues following topical administration of MDA on mice skin. This was an unusual outcome because the tumors arose in a very quickly and were not the kind of tumors generally

observed in a mouse skin assay (Marnett 1999). Up now, there are several experiments and trails avaiable showing the carcinogenetic properties of MDA; the most significant will be illustrated in the continue of this dissertation (see "MDA and cancer"section).

MDA and other human pathologies.

According to Del Rio, who had published an interesting review about MDA in 2005, MDA is able to impair several physiological mechanisms of the human body through its ability to react with lots of molecules, not only DNA but also proteins.

In the last 20 years, the amount of MDA levels in biological samples from subjects affected by several diseases, besides cancer, has been widely measured and MDA levels have been used as a common oxidative stress biomarker MDA toxicity is not only involved in cancerogenity mechanisms, but also towards cardiovascular stability. MDA reacts in vivo with primary amines to form the N3-(2-propenal)lysine and generate lysine-lysine cross-links with 1-amino-3-iminopropene and pyridyledihydropyridine type bridges. These reaction compounds have been found out in apoB fractions of oxidized lipoproteins (LDL) and are considered to be implicated in the impaired interaction of the modified lipoproteins and macrophages. This phenomenon could be the start up of atherogenicity.
Another probable toxic action of MDA concerns collagen. Even if the nature of the cross-link is not yet explained in detail, the inter-molecular cross-linking of collagen through MDA may significantly yields to the stiffening of cardiovascular tissue . Polidori and collaborators found lower levels of antioxidants and higher levels of MDA in congestive heart failure patients comparing to healthy subjects in a case control study subjects. Plasma from atherosclerotic patients was richer in MDA than controls in a study by Tamer and co-workers.
Prevalence of cardiovascular diseases in haemodialysis patients has also been analyzed in relation to oxidative stress. Serum MDA values were markedly high in haemodialysis patients and higher in haemodialysis patients with cardiovascular complications in two studies reported by Boaz and colleagues. Haemodialysis patients were found to be more oxidatively stressed in particular in the posthaemodialysis phase when compared to healthy controls by Ozden and colleagues .

Pre-eclampsia, a disease tipical of pregnancy and characterized by hypertension, proteinuria and generalized edema, has been analyzed by Yoneyama and collaborators who have connected plasma MDA levels to adenosine deaminase ones (ADA-a marker of T-cell activation, in this pathology). They noted that levels of MDA in plasma from patients suffering pre-eclampsia were markedly and significantly higher when compared to plasma from patients enjoying a normal pregnancy. Another correlation examined in pre-eclampsia is between MDA and ceruloplasmin activity. Orhan and colleagues explained this correlation with the possible response of the body to increased oxidative stress. Besides, values of MDA were found to be significantly higher in conditions of pre-eclampsia as compared with healthy pregnancies and values in uncomplicated pregnancy were higher than in non-pregnant controls. High values of MDA in pre-eclamptic patients as well as the hypothesis of increased oxidative stress in uncomplicated pregnancy were confirmed by Ilhan et al .

Diabetes is one of the more common pathologies that lead to oxidative insult in its etiology and complications. MDA plasma levels were observed to be higher in non-insulin dependent diabetes mellitus (NIDDM) subjects than in healthy controls in a study carried out by Dierckx and colleagues.

The research group also found that MDA levels were linked to glutathione and uric acid, but only in diabetic subjects. Additionally to diabetes, even subclinical complications, such as neuropathy, nephropathy, or retinopathy could have a role in growing the levels of MDA.In diabetic patients with subclinical complications studied by Martin-Gallan group, MDA levels resulted higher than in a matched control group with diabetes, but without any complications.

As concerning liver diseases, there are studies that attested that plasma MDA concentrations were higher in alcoholics, with or without cirrhosis, and in viral patients cirrhosis than in matched healthy controls.

In addiction, higher levels of MDA have been observed in Alzheimer's type dementia patients and also in acute abdominal pain. (Del Rio 2005).

Detection of MDA

To determine MDA, most assays have been elaborated on the basis of its derivatization with thiobarbituric acid (TBA). The condensation of these two molecules (MDA and TBA) leads to the formation of a high absorbivity, pink adduct, (consisting in two mol of TBA and one mol of MDA), which can be assessed with a spectrophotometer (Jetawattana 2005, Del Rio 2005)

Unfortunately, the specificity of the test based on this reaction is low, as TBA can react with other several molecules rather than MDA. Moreover, the treatment of biological samples to obtain the condensation compound is usually carried out at high temperature (around 100 C) and acidic condition; this enviroment may give further oxidation of the matrix with conseguent overestimation of the results. To reduce matrix oxidation, most of these methods involve the precipitation of protein prior to the TBA reaction as a pre-treatment of plasma samples.

One of the first and still most widely used methods to detect MDA has been developed by Yagi, carrying over the TBA reaction on a blood lipid and protein precipitate at 95 C in acidic conditions. These results are often reported as ''TBA reacting substances'' (TBARS) instead of MDA. Plasma MDA or TBARS concentrations, measured with the methods developed from 1970 to 1995, confirm the sample oxidation during analysis but varied in a very wide not reliable range of results. Thus, even if TBARS measurements often demonstrate levels of oxidative stress higher in pathological than in healthy conditions it is important to remember that TBARS assays gives an idea of sample oxidizability, rather than sample oxidation and that the results obtained with this type of method should be carefully analysed before any conclusions are drawn.

Recently, several innovations have been introduced to increase the specificity and to avoid the common biases of the old procedures.

It is important to consider the difference between free and total plasma MDA.
Little is known about the pathophysiological significance of the two forms, but their different chemical features yields to a different chemical approach of extraction. Free MDA is unbound to plasma protein or any other biomolecule and because of this, it can be measured without any hydrolytic sample treatment. The significant amount of MDA bound to matrix molecules, is undetectable without an adequate physico-chemical step to liberate it. To reach this result, it is possible to use acidic or basic sample pre-treatments in the presence of protein, or strong acids to precipitate the protein fraction. Techniques which avoid sample pre-treatment or where precipitation of protein is obtained by means of organic solvents, are considered as non-hydrolytic and can test only the free form of MDA. Unless otherwise

specified, TBA-based methods are usually centred on total MDA detection, mainly due to their strong temperature and acidic conditions of analysis. In the next figure, the most recent and precise methods to assess MDA in human plasma or serum are briefly described.

Table 1 Reference, derivatization agent, brief description, mean values ($\pm$standard deviation), sample volume (SV) used and blood anticoagulant (AC, where indicated) of methods applied in recent years for malondialdehyde determination

Reference	Derivatization	Description	MDA (μmol/L)	SV (μL)	AC
Templar et al. (1999) [16]	TBA	Acidic deproteinization, HPLC-UV/Vis	0.11±0.03	250	EDTA
Agarwal and Chase (2002) [18]	TBA	HPLC-fluorimetric	0.69±0.13	50	–
Del Rio et al. (2003) [19]	TBA	Mild reaction conditions, fluorimetry	0.112±0.034	200	EDTA
Karatas et al. (2002) [22]	None	Mild acidic deproteinization, HPLC-UV/Vis	0.50±0.04	50	No (serum)
Wilson et al. (1997) [23]	None	Acetonitrile deproteinization, HPCE	n.d.	500	Heparin
Sim et al. (2003) [24]	DNPH	Strong acidic deproteinization, HPLC-UV/Vis	13.8±1.32	50	EDTA
Steghens et al. (2001) [25]	DAN	Strong acidic conditions, HPLC-UV/Vis	0.162±0.051	100	Heparin
Cighetti et al. (1999) [26]	PH	Strong acidic conditions, GC-MS	1.3±0.07	200	EDTA
Stalikas and Konidari (2001) [28]	TCPH	Mild conditions, GC-MS or GC-ECD	0.48±0.03 (ECD); 0.50±0.03 (MS)	–	–
Cighetti et al. (2002) [27]	PH	Mild conditions, GC-MS (ID-GC-MS validated)	1.41±0.23	200	EDTA

n.d., not detectable; TBA, thiobarbituric acid; DNPH, 2,4 dinitrophenylhydrazine; DAN, diaminonaphtalene; PH, phenyl-hydrazine; TCPH, 2,4,6-trichlorophenylhydrazine; HPLC-UV/Vis, high performance liquid chromatography with ultraviolet or visible light detector; HPCE, high performance capillary electrophoresis; GC-MS, gas chromatography-mass spectrometry; GC-ECD, gas chromatography-electron capture detection; ID-GC-MS isotope dilution-gas chromatography-mass spectrometry.

Figure 7. MDA detection (Del Rio 2005)

The method of Templar and colleagues consists on a optimized high performance liquid chromatography method with ultraviolet-visible detection (HPLC-UV/Vis) providing separation and identification of the MDA-TBA adduct. There is the fully validated deproteinization step, which excludes the column wash without any loss of sensitivity. All the analytical parameters (linearity of the method, detection limit, precision, sample stability and interferences) are adequate and the values obtained for plasma MDA in healthy subjects as well as in patients are between 0.1- 1 mmol/L. Anyway, the MDA-TBA reaction conditions still involve incubation at high temperature (90 C for 45 min) and the addition of BHT (butyl hydroxytoluene) could be not enough to avoid matrix oxidation.

Another method still using TBA has been developed by Agarwal and Chase and it is based on HPLC (high performance liquid chromatography method) with fluorescence detection. The protein precipitation step is skipped. Avoiding protein precipitation permitted the analysis of MDA covalently bound to proteins, even if precipitating proteins give a better chromatographic signal and prolong the lifespan of the column when a chromatographic separation is involved. This method is very analytically performing and

chromatographically quick. The derivatization with TBA is still the weak step, as it occurs again in conditions of high temperature, and the limit of detection is high as compared with other equivalent techniques.

The problem of maintaining or skipping the protein precipitation step is completely overcome in the third method based on TBA reaction, used by Del Rio and colleagues and following illustrated. This method is based on the observation that the MDA-TBA adduct interacts with albumin and this interaction may lead, in appropriate conditions, to a strong increase in its natural fluorescence. In the light of this signal increase, the plasma sample pre-treatments are reduced to a minimum.

Briefly, 200 mL of plasma are allowed to react with TBA in acidic conditions at 37 C for 65 min and an aliquot of the reaction mixture is then transferred to the spectrofluorimetric cuvette. The measurement arises from the addition of an excess of bovine serum albumin in acidic pH. The method is fully validated and the limits of detection and quantification are 0.015 and 0.025 mmol/L, respectively. The mild reaction conditions allow a minimum interference of coreacting species and at the same time a drastically reduced overestimation of the analyte, even in the absence of a chromatographic separation,

Instead the Japanese group of Sheu and collegues successfully applied an online microdialysis system together with an HPLC (high performance liquid chromatography method) equipped with a UV-Vis (ultraviolet/visible) detector to quantify the MDA-TBA adduct. The derivatization reaction happens at high temperature, but the sample at that stage should be depleted of all the potential oxidizable matrix such as fats and protein. Unluckily, the system has only been applied to an in vitro peroxidation model. .

Method without derivatization and new derivazing agents have been tested, to overcome the biases linked to derivatization of MDA with TBA.

A method that does not use any derivatization has been ideated by Karatas and colleagues. The method limits plasma sample pretreatment to the acidic hydrolysis of amino acid-bound to MDA and consequent protein precipitation. The protein-free plasma is directly injected onto an HPLC (high performance liquid chromatography method) and the very quick elution of MDA (monitored at 254 nm) allows very rapid analyses. The limit of detection of this method is relatively low (0.012 mmol/L), the linearity is maintained over a very satisfying range of concentrations and the chromatogram quality is high. Another advantage introduced by this method is the very low amount of plasma needed for the analysis (50 mL).

Wilson and colleagues also analysed in plasma MDA without derivatization method by the use of high performance capillary electrophoresis (HPCE). The method validation is very accurate and the precision is high. The sample pre-treatment includes only the precipitation of the protein fraction with acetonitrile, thus allowing the potential detection of only free MDA and leaving untouched the fraction bound to protein or other biomolecules present in plasma.

The HPCE tracings obtained by supplemented plasma are very clear and no confounding peaks appear to influence the quantification. However, no detectable MDA is found in any of the analysed plasma samples with this method, even if a parallel TBA assay and a commercially available colorimetric assay (LPO- 586, Bioxytech) can detect a marked amount of analyte in the same sample fractions. Besides the HPCE method is referred as ten-fold more sensitive than an HPLC in this analysis, therefore the absence of significant signals is interpreted as a real absence of free MDA in plasma.

Among methods that use new derivatizing agents, HPLC or gas chromatography (GC) are the most common techniques applied (Sim and colleagues used 2,4-dinitrophenylhydrazine, DNPH; Steghens and collegues used diaminonaphthalene, DAN; Cighetti and colleagues

used phenylhydrazine, PH; Stalikas and Konidari used 2,4,6-trichlorophenylhydrazine, TCPH)

Instead Suttnar and colleagues studied the influence of anticoagulants used to preserve blood samples from coagulation.. They reviewed the literature avaible about this topic and observed the different effect of citrate and EDTA on the final plasma MDA concentration. It appeared that the anticoagulant chosen has great influence on MDA values detected, and that the use of EDTA instead of citrate results in lower MDA concentration.

MDA can be found also in other biological samples besides the blood.

Larstad and her group developed a test to determine MDA in breath condensate by means of HPLC(high performance liquid chromatography method) with fluorescence detection. The sample collection is made by a breath condenser and the method is based on TBA HPLC determination.. However, due to the minor complexity of the matrix considered for analysis, the strong reaction conditions should not constitute a bias in this case. The average reported concentration for MDA in breath condensate of healthy subjects is 0.15 pmol/s. It should be considered that increased MDA in breath condensate might reflect air way inflammation

Urine has also been used as a biological sample for MDA detection by some authors.

Agarwal and Chase applied their method (which has been described above) also to urine. Values obtained for normal healthy subjects range from around 2 mmol/mg creatinine.

Korchazhkina and colleagues described a method to assess MDA in human urine after derivatization with DNPH (2,4 dinitrophenylidrazine). The average concentration of urinary MDA was ranging around 0.019 mmol/mmol creatinine. Interestingly a parallel experiment, carried out using a classical TBA assay, resulted in ten-fold higher MDA levels recoveries, thus indicating that, even in a virtually lipid and protein-free sample like urine, strong reaction conditions and non-specificity of TBA may play a significant role in analyte overestimation.

The main limitation of this method is the marked matrix effect due to the interference of urea. This matrix effect forced the researchers to calibrate the method and analyse the samples with the time consuming standard additions method.

Detection of MDA: DNA adducts

Specific assays have been developed to analyse MDA-DNA adducts. The main target of these analyses is the most common adduct M1dG. Mass spectrometry is often used in DNA adduct determination.

Doerge and colleagues developed a method based on the HPLC (high performance liquid chromatography method) separation and tandem mass spectrometric to identify and quantify MDA and other aldehydes in the form of their adduct with 2- deoxyribonucleotides in human and animal tissues.

Otteneder and collaborators studied in detail the reactions of the adduct M1G and hydrazines. They observed that M1dG rapidly reacts with pentafluorobenzylhydrazine (PFBH) to give pentafluorobenzylpyrazole (PFBP) and deoxyguanine (dG). Based on this observation, the group developed an assay based on GC-MS (gas cromatografy-mass spectrometry) to quantify M1dG in DNA samples .

Rouzer and colleagues developed a GC-MS (gas cromatografy-mass spectrometry) method, which couples electron capture detection and tandem mass spectrometry, to detect, identify and quantify M1dG in DNA isolated from the blood of healthy donors

Hakala and colleagues used HPLC coupled with tandem mass spectrometry to detect and quantify M1dG in tissues, using an analogue stable isotope as internal standard.

Yi and colleagues detected M1dG with 32P-postlabelling HPLC method. This kind of detection is recognized as the gold standard for detection and quantification of carcinogen-modified DNA adducts.

Leuratti and her group described the immunoslot blot (ISB) assay for the measurement of M1dG. The main advantage of the ISB assay is the minimum sample amount required and the fact that it is less time consuming and laborious with respect to chromatographic techniques.

Ottender and collegues tried to assess M1dG also in urine. Unfortunately, no detectable amount was found in urine from rats, even when treated with CCl4, a very common oxidative stress inducing agent.

Anyway methods of dection are being continuously developed to improve identification and quantification of MDA or its adduct with biological molecules. (Del Rio 2005)

The method used in our laboratory to detect DNA adducts is based on reversed-phase HPLC, mass spectrometry and 32P-postlabelling assay and it will be explain in detail in the section "Laboratory measurement adducts".

MDA levels in humans between lifestyle and environment

Literature review

To understand how it is possible to prevent the formation of MDA and consequently the chain happening that this formation yields, is needed to study the existing linkage between MDA and lifestyle and environment.

We have reviewed the literature about this topic until December 2012. We used Pubmed (www.nbci.nml.nih.gov/pubmed) as search engine. We tried many research keys. We focused on observational studies or intervention trials conducted on humans, excluding those on animals.

Authors	Population	Year of publication	Area	Diet	Physical activity	Work / Stress / Life style	Smoke	Alcohol	Obesity
				Main topics analyzed					
Peluso*	187	2012	Thailandia	x		x			
Sun*	124	2012	Germany	x					
Egert	74	2012	Germany	x					
Kurtul	100	2012	Turchia				x		
Karbowink-Lewinska	78	2012	Polonia						x
Sankla	150	2012	India						x
Codoner-Franch	40	2012	Spain						x
Cardoso	34	2012	Brasile		x				
Somannavar	100	2012	India	x					
Azzini	131	2011	Italy	x					
Potter	17	2011	USA	x					
El Abed	20	2011	Tunisia		x				
Kozirog	63	2011	Poland						
Casado	102	2011	Spain	x	x	x	x		
Kim	976	2011	Korea	x					
Tonguc	85	2011	Turkey				x		
Peluso*	173	2010	Thailandia	x		x	x		
Leelarungrayub	24	2010	Thailandia		x				
Caimi	241	2010	Italy	x	x				
Zemel	20	2010	n.d.	x					x
Botelho	57	2010	Brazil	x					
Pooya	82	2010	Iran	x					
Basu	35	2010	USA	x					x
Munoz Marin	20	2010	Spain		x				
Alvarez	29	2009	Spain	x	x			x	
Bloomer	30	2009	USA	x					x
Mergener	110	2009	Brazil		x				

Authors	Population	Year of publication	Area	Diet	Physical activity	Work / Stress / Life style	Smoke	Alcohol	Obesity
Casado	67	2008	Spain	x	x		x	x	
Komatsu	365	2008	Japan/Mongol	x					
Jinam	n.a.	2008	Malesia						x
Valtuena	33	2008	Italy	x					
Zawadziski	104	2008	Poland				x		
Guentsch	60	2008	Germany				x		
Kelishadi	35	2008	Iran	x	x				x
Sano	61	2007	Japan	x					
Li	90	2007	China				x		
Bloomer	28	2007	USA	x			x		
Babu	100	2006	India			x			
Tsuboi	33	2006	Japan			x			
Staruchova	388	2006	Slovenia	x					
Komatsu	384	2006	Japan/Mongol	x					
Bamonti	32	2006	Italy	x			x		
Zhu	170	2006	China	x					x
Yadav	104	2005	India	x		x			
Anlasik	87	2005	Germany	x					
Nagao	35	2005	Japan	x					
Yuksel	54	2005	N.d.					x	
Venkatesan	25	2005	India				x		
Solak	130	2005	Turkey				x		
Celec	232	2005	Slovenia				x		
Basyigit	30	2005	Turkey				x		
Franzoni	64	2005	Italy		x				
Brufau	47	2004	Spain	x					
Ermis	43	2004	Turkey				x		
Lykkesfeldt	80	2004	Denmark				x		
Mircea	32	2004	Romania				x		
Lasheras	162	2003	Spain	x					
Metin	n.a.	2003	Turkey		x				
Lawler	34	2002	USA		x				
Georgieva	69	2002	Bulgaria			x			
Engstrom	16	2002	North Europe	x					
Stewart	39	2002	USA	x					
Kleemola	95	2002	Finland	x					
Vassalle	54	2002	Italy				x		
Ozbay	257	2002	Turkey		x		x		
Aluntas	n.a.	2002	Turkey				x		
Hagenlocher	42	2001	Germany	x					
Trevisan	1797	2001	USA			x	x	x	
Rust	60	2001	Austria	x			x		

Authors	Population	Year of publication	Area	Main topics analyzed					
				Diet	Physical activity	Work / Stress / Life style	Smoke	Alcohol	Obesity
Kharb	55	2001	India				x		
Kim	n.a.	2001	Korea	x			x		
Rolla	145	2000	Italy					x	
Codandabany	60	2000	India				x		
Ayaori	149	2000	Japan				x		
Freese	20	1999	North Europe	x					
Maskarinec	29	1999	USA	x					
Meagher	92	1999	USA					x	
Sturmen-Gur	nd	1999	Turkey				x		
Hernandez	44	1999	Spain		x				
Dixon	9	1998	USA	x					
Beer	1397	1998	France	x		x	x	x	
Marangon	459	1998	France	x			x		
Nielsen F	213	1997	Denmark				x	x	
Nair	20	1997	North Europe	x		x	x	x	
Akkus	108	1997	Turkey					x	
Miller	123	1997	USA				x		
Fang*	59	1996	Finland	x					
Lecomte	619	1994	France					x	
Wakabayashy	114	1994	Japan	x					
Nelson	9	1993	USA	x					
Baldi	80	1993	Italy					x	
Gatti	25	1993	Italy					x	
Hoving	24	1992	Netherlands	x					
Wakabayashy	67	1992	Japan	x					
Knight	378	1987	USA			x			

Table 1. *MDA and its determinants (literature review). Note: *papers measuring MDA-DNA adducts.*

The previous table summarizes the studies available in International Literature about the existing linkage between MDA and lifestyle and environment, according to the previous research strategy. The table reports the first author name of each paper analyzed, the year of publication, the country in which the study was conducted in the world, the main topic of each study and the people recruited. The majority of the studies were carried out in Europe (43%). What appears clear since a first look, is that the population enrolled in each reviewed study, is mostly quite small (the number average of the enrolled population is 142, and the median is 67).The frequently limited sample size of the studies could at least partially explain the not univocal results found.

Only few of the studies reviewed measured MDA-DNA adducts (Peluso 2012, Peluso 2010, Sun 2012, Fang 1996). The study we have conducted and that we are going to describe in

the fourth chapter, is based on the measurement of MDA-DNA adducts and we are going to explain more extensively this topic in the last section of this paper.

Diet

Food consumption has been going studying since a long time. This interest arose very early, because food appeared to be in a very close linkage with damages caused by lipid peroxidation products. In particular saturated fats and dietary cholesterol, because of their supposed role in the etiology of atherosclerosis and ischemic heart disease, have received an inordinate amount of attention in relation to public health (Kanner 2007).

In 1993 Esterbauer published an interesting review about lipid peroxidation products, including also this topic, focusing on the large number of experimental animal studies that had been conducted during the previous years in order to assess the potential health risk of food rich in lipids.

The conclusion that appeared from the studies that Esterbauer had analyzed, is that heavily oxidized oils given orally are not acutely toxic. Upon chronic feeding of such materials, however, rats react with growth retardation, intestinal irritation enlarged liver and kidney, haemolytic anaemia, increased serum concentrations of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), decreased amounts of vitamin E in serum and liver and increased lipid peroxides in the liver, as measured by thiobarbituric acid-reactive substances (TBARS). A possible explanation for the unexpectedly low acute toxicity of heavily oxidized oils and fats is that di- and polymeric-oxidation products are not well absorbed in the intestine and therefore do not reach directly the blood stream. Furthermore, peroxides are detoxified by glutathione-dependent enzymes in the gut to less toxic lipid alcohols, which then appear in the various organs.

Low-molecular- weight aldehydic lipid peroxidation products such as hydroperoxy-alkenals or hydroxyalkenals, are more readily absorbed than other polymeric oxidation products and lipid hydroperoxides and produce several pathological effects such as damage to liver, thymus, and kidney. Anyway humans ingest significantly lower quantities of frying oils and lipids than those used in animal studies. In his review Esterbauer pointed the attention particularly on lipid peroxidation tumor-promoting effect, reviewing animals studies. In several studies it was reported that chronic oral administration of MDA to mice in the drinking water or application to skin followed by a tumor promotor (two-stage application) increases tumor frequency in mice.

Esterbauer reported in his review that lipid hydroperoxides could have a tumor-promoting effect because they stimulate cell proliferation in the colon and that the chronic inhalation of cooking vapors containing volatile lipid-oxidation products, such as acrolein and other aldehydes, could be a potential risk for lung cancer. There were also animals studies that didn't find any correlations.Esterbauer reported also lots of previous animals studies that demonstrated a strong linkage between atherosclerosis and coronary diseases, and assumptions of rich in lipids food (Esterbauer 1993).

The interest about this topic is still deep. Many epidemiological studies infact, as well as experimental data, indicate that populations on diets characterized by the Western pattern, with high intakes of high-fat red-meat, processed meat, butter, processed and fried foods, eggs and refined grains, and low in fruits and vegetables, are at a high risk for the development of atherosclerosis and of several kinds of cancers, especially colon cancer. With regards to the chemical constituents, the Western diet includes large quantities of oxidized fatty acids, oxidized cholesterol, cytotoxic aldehydes, and phospholipids, because a

large proportion of the food in the diet is often consumed in a fried, heated, processed, and long stored form.

Lipid oxidation in foods in one of the major degradative processes responsible for changes in flavour, colour, and texture; the oxidation of unsaturated lipids results in significant generation of cytotoxic and genotoxic compounds Furthermore, the free radicals produced by the process of lipid peroxidation not only generate cytotoxic compounds but also co-oxidize vitamins such as vitamin A and carotenoids, vitamin E and vitamin C, and thereby impair the nutritional quality of the foods. MDA is the most abundant individual aldehyde that results from lipid peroxidation in foods. Its concentration in meat and fish products could reach 300 µM or more. Further complexity is added considering that dietary fatty acids and dietary cholesterol are co-oxidized. The gastrointestinal tract is constantly exposed to dietary oxidized food compounds generated during the reactions that take place during processing and storage of foods.

There is the hypothesis that the stomach and its gastric fluid could be a medium for further dietary lipid oxidation or antioxidation. The stomach receives the masticated food and from time to time is open to the atmosphere; at least during the meal time, it acts in an aerobic environment. Red meat, homogenized and incubated in human gastric fluid, exhibited a further auto-oxidation process that generated LOOHs (hydroperoxides, 2000–5000 µM after 180 min) and MDA (120 µM). The crossreaction between free radicals, produced during this reaction, co-oxidized vitamin E, β-carotene, and vitamin C.

However, some authors proposed that antioxidant and other protective effects of phenolic compounds can occur inside the gastrointestinal tract itself. Indeed, it seems that both lipid peroxidation, generation of advanced lipid peroxidation endproducts and co-oxidation of the vitamins in stomach medium could be inhibited by food polyphenols.

Other authors described the dual role of saliva in lipid peroxidation under stomach conditions. It was found that lactoperoxidase increases lipid peroxidation, whereas thiocyanate and, especially nitrite, in the presence of reducing compound, reduce it. The inhibitory effect of nitrite is due to nitric oxide. Anyway further studies about the antioxidant effect of saliva on co-oxidation of vitamin E in gastric fluid, demonstrated that saliva alone can not protect against the cooxidation, and that the presence of polyphenol antioxidants is required. Nevertheless, the reactions that take place in the stomach seem to be of great importance because the breakdown of hydroperoxides products can lead to generation of not toxic or very toxic compounds.

Glutathione (GSH) is abundant in the mucosal cells of the GI tract, and the exogenous introduction of GSH from foods changes its concentration; its bioavailability from the ingested food depends on the intake of dietary proteins, especially sulfur amino acids. A standard human diet contains about 150 mg/day of GSH; its concentration in the gastrointestinal tract could be affected by oxidized reaction products derived from oxidized foods or reactive aldehydes. Reactive aldehydes such MDA are known to react spontaneously with glutathione (GSH).

The gastrointestinal GSH represents a first line of defence against ingested hydroperoxides, reducing the amount of hydroperoxides transported from gut into the lymph. Anyway after digestion, a part of the dietary oxidized food compounds, are absorbed into the lymph or directly into the blood stream. After ingestion of oxidized fats, animals and human have been shown to excrete in urine larger amounts of malondialdehyde but also lipophilic carbonyl compounds.

After absorption these compounds can react with other molecules following the pathways mainly described above in this introduction, potentially provoking many damages. The consumption of oxidized fat in the diet represents a chronic threat to human health, while a diet containing large amounts of dietary antioxidants such those present in fruit and vegetables, or products such as red-wine or tea, represents an health protection. (Kanner 2007).

Besides food eaten, cells continuously produce free radicals and reactive oxygen species (ROS) as part of metabolic processes. These free radicals are neutralized by an elaborate antioxidant defense system consisting of enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and numerous non-enzymatic antioxidants, including vitamins A, E and C, glutathione, ubiquinone, and flavonoids (Urso 2003). Antioxidant supplements are marketed to and used especially by athletes as a means to counteract the oxidative stress of exercise. It is not clear if a surplus antioxidants could be useful in those conditions or in similar ones to balance an eccess of ROS. (Urso 2003, Clarkson 2000)

Concerning our review of the literature, the studies we analyzed, broadly agree with the concepts we have here described.

Topic	MDA =	MDA ↑	MDA ↓	TOTAL
Red wine	0/1	0/1	1/1	1
Potatoes	0/1	1/1	0/1	1
Vitamin E	0/1	0/1	1/1	1
Dairy products	0/1	0/1	1/1	1
Proteins eaten	0/1	1/1	0/1	1
Carbohydrate	0/1	0/1	1/1	1
Fruit	1/10	0/10	9/10	10
Vegetables	1/12	0/12	11/12	12
Fried fruit	1/1	0/1	0/1	1
Grilled food	1/1	0/1	0/1	1

Table 2. *MDA and diet in literature review.*
Note: "MDA =/ MDA ↑/MDA ↓" refers, for each food item, to the number of the most important reviewed studies where MDA levels result respectively "the same/more/less" in the study sample compared with the control group or in the same study group after the experimental intervention.

The study carried on by Kim and colleagues enrolled 976 adults between April and December 2005 in Seoul, Corea. They assessed the relationships between relatively simple dietary quality scores modified for a Korean diet (such as the Recommended Food Score (RFS) and alternate Mediterranean Diet Score (aMDS)), and oxidative stress biomarkers in Korean adults. RFS and aMDS were calculated by using a food-frequency questionnaire; regression analyses assessed the associations between diet quality scores and urinary malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG).
While there were no significant associations of RFS and aMDS with urinary 8-OHdG concentrations. RFS and aMDS were negatively associated with urinary MDA concentrations; it means that MDA levels were lower in individuals having a diet rich in fruit and vegetable and poor in lipids. After stratified analyses by sex, negative associations between the both scores and urinary MDA concentrations were not significant in both men and women. (Kim 2011)

Staruchova and colleagues confirmed that a balanced food consumption, with higher fruits and vegetables intake, has a protective effect against oxidative damage. They conducted a molecular epidemiological study in three Slovak factories, investigating 388 subjects (239 exposed to factory pollution, 148 not exposed controls).

Food frequency questionnaire was used to achieve the information of nutrient intake; the nutrient intake was compared to plasma levels of selected micronutrients, as well as to markers of oxidative stress (MDA, oxidative DNA damage and DNA repair) and antioxidant protection. They found an inverse and significant correlation between MDA concentrations and consumption of fruits and vegetables in all control subjects. Intake of fruits, vegetables , milk and cereals inversely correlated with oxidative DNA damage in all subjects investigated. (Staruchova 2006)

Likewise Sommanavar conducted a study comparing a group of 50 vegetarian healthy people, to another composed by 50 non-vegetarian healthy people. He verified significantly higher MDA levels in the group composed by non-vegetarian people. (Somannavar 2012)

Two interesting study have been carried on by Komatsu and colleagues in 2006 and 2008. They have quite the same structure and arrived to overlapping conclusions. In the second and more recent one, the food intake inquiry, anthropometric measurements and blood clinical tests were performed for 365 healthy inhabitants in Murun, a northern Mongolia city, and compared to those of Japanese, in order to understand Mongolians relatively short life expectancy, examining the role of dietary habits in the early aging of Mongolians. The results they obtained, suggested that the Mongolians dietary habits are associated with their lifestyle-related diseases and early aging, and that the improvement of dietary habits may be an effective strategy for health promotion of the inhabitants. Indeed Murun inhabitants were found to have a characteristic dietary habit of taking large amounts of meat, milk, dairy products and wheat flour products, in contrast to little vegetables, fruits and fishes.

The daily calorie intake of the adults was estimated to be 2,525 kcal, and the fat/total calorie ratio was calculated 33.7%, about 1.3-fold higher than that of Japanese. The intake ratio of fatty acid from the Mongolian foods, saturated : mono-unsaturated(MUFA) : poly-unsaturated fatty acids (PUFA) ratio, was 10.3 : 7.8 : 3.0. Results of blood clinical tests showed significantly higher levels of serum triglycerides, low-density lipoprotein cholesterol (LDL) and homocysteine, and lower levels of high-density lipoprotein cholesterol (HDL), n-3 PUFA, folic acid and adiponectin, in comparison with those of Japanese. In addition, the Mongolians were also found to have significantly high levels of oxidative stress markers, such as serum malondialdehyde-modified LDL (MDA-LDL), urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) and serum reactive oxygen metabolites (ROM). Obesity was observed at a high incidence in the subjects over 30-year old. (Komatsu 2008).

Instead Anlasik and colleagues pointed their attention on a elderly population to evaluate whether the often-reported age-related decrease of plasma antioxidants in humans depends on differences in dietary intake or on other age/gender-related factors. They compared forty-eight healthy subjects aged 65 and older consuming low intakes of fruit and vegetables daily with thirty-nine community-dwelling healthy subjects aged 65 years and older consuming high intakes of fruits and vegetables daily .

Plasma levels of retinol, tocopherols, carotenoids and malondialdehyde as well as content of protein carbonyls in IgG were measured. Plasma levels of retinol(vitamin A), tocopherols and carotenoids were significantly higher in group that have a diet rich in fruit and vegetables than the other, independently of age and gender. MDA levels were inversely correlated with vitamin A and α-carotene. (Anlasik 2005)

Another study focusing on Mediterranean Diet Score is an Italian study, promoted by Azzini and colleagues. It was an observational study conducted on 131 healthy free-living subjects. Dietary intake was assessed by dietary diary. Standardised procedures were used to collect anthropometric measurements.

Antioxidant status (vitamin A, Vitamin E, carotenoids, vitamin C, uric acid, Sh groups, SOD and GPx activities), total antioxidant capacity (FRAP and TRAP), lipid blood profile (total/HDL/LDL cholesterol, triglycerides), immune status (TNF-α, Il-10 cytokines), lipid peroxidation marker (MDA in the erythrocytes) were evaluated on blood (serum, plasma and whole blood). The adherence to a Mediterranean dietary pattern was found to be associated with a better cardiovascular profile, reduced oxidative stress and modulation of inflammation. Regarding to malondialdehyde, lower levels of MDA were associated with a high adherence to the Mediterranean Diet (Mediterranean Diet Score > 4) (Azzini 2011).

Peluso and colleagues studied MDA adducts instead of MDA circulating levels, analysing the association between diet and M1dG in 67 industrial estate workers in Map Ta Phut, Rayong, Thailand and in 120 controls (a sample population of a close area).

The strong influence of fruit and vegetables assumption was found (M1dG resulted decreased in controls reporting to consume 14–17 servings/week of fruit and vegetables) Instead it was not detected the expected significant M1dG increment in controls consuming 9–18 servings/week of fried food (there was only a not significant increment) and no increment in MDA levels was found in those people, between controls, who usually eat charcoal-grilled/barbecued food. (Peluso 2012)

Moreover there are very interesting studies in literature, regarding the linkage between lipids and MDA levels.

Hoving and colleagues reported in 1992 a study on 24 individuals, emerging the correlation between plasma triglycerides, total cholesterol, total fatty acids eaten and the total number of double bonds present in plasma fatty acids characterized by three or more doube bonds. In the years after, more articulated studies, probing the relation between fatty acids diet assumption and MDA levels, have been organized. Briefly, the main conclusion is that the diet assumption of PUFA yields to an over-production of MDA adducts.(Hoving 1992)

Nair reported in 1997 that high intake of ω-6 polyunsaturated fatty acids (ω-6 PUFA) increased malonaldehyde-derived adducts in male and female subjects. Twenty volunteers consumed a milk fat-based diet that was rich in saturated fatty acids for 14 days.

Following this initial period, after which the group was considered homogeneous with respect to diet, 9 subjects were allocated for 25 days to either a sunflower *(Helianthus annuus)* oil-based diet that was rich in PUFAs (fatty acids content of the oil: 12% saturates, 23% monounsaturated, 65% ω -6 polyunsaturated, and traces of ω-3 polyunsaturated) and eleven to a low erucic acid Finnish turnip rapeseed *(Brassica rapa ssp. oleifera DC)* oil-based diet rich in MUFAs **(**fatty acids content of the oil: *5%* saturates, *58%* monounsaturated, 24% *ω-6* polyunsaturated, and 13% ω-3 polyunsaturated of the total fatty acids. (Nair 1997).

The year before Fang and colleagues conducted a similar experiment on a group of 59 healthy individuals. They were fed, for 14 days, with a milk fat based diet rich in saturated fatty acids. Following this initial period, after which the group was considered homogeneous with respect to diet, 30 randomly chosen subjects were given a sunflower oil-based and 25 days long diet (rich in polyunsaturated fatty acids-PUFAs); the remaining 29 individuals were given a low erucic acid rapeseed oil-based and 25 days long diet (rich in monounsaturated fatty acids).

The fatty acid composition of plasma lipid fractions and the level of MDA-DNA adducts in total white blood cells were determined at the end of this period. Higher concentrations of polyunsaturated fatty acids in plasma triglycerides and higher levels of MDA- DNA adducts were found in the subjects following the PUFAs diet when compared with those of the MUFAs dietary group. (Fang 1996)

Later in 2012, Sun and colleagues analyzed DNA extracted from buffy coat and plasma samples of 124 healthy women participating in the EPIC-Heidelberg cohort study. Positive correlations existed between M1dG levels and linoleic acid intake and between M1dG levels and the ratios of dietary linoleic acid/oleic acid and PUFA/MUFA. (Sun 2012)

There are then some studies available in literature confirming the circulating MDA levels reduction when some anti-oxidants products are included in the diet at the adequate quantities. (Potter 2011, carrot juice; Kozirog 2011, melatonin; Alvarez 2009, alcohol beer free; Bloomer 2008, vitamin C; Sano 2007, grape seed- 72% proanthocyanidin; Brufau 2004 alpha-tocopherol and beta-carotene). Anyway these studies, even if interesting, don't yields really significant results, because the authors analyzed different substances and tested them on little sample population.

Basu and colleagues and Nagao and colleagues confirmed the antioxidant properties of green tea, testing in their trials the reduction of circulating MDA levels thanks to the regular assumption of green tea. (Basu 2010, Nagao 2005)

In literature there are also some articles that show contrasting results, in comparison to the previous exposed biochemical pathways and of the previous described studies.

Valtuena and colleagues, analyzing the results from their crossover intervention trial on 33 healthy individuals, concluded that selecting foods according to a dietary total antioxidant capacity, markedly affects antioxidant intake and modulates hepatic contribution to systemic inflammation without affecting traditional markers of antioxidant status. Moreover they found unexpectedly that MDA circulating levels decrease significantly with a low antioxidant capacity diet (Valtuena 2008)

Zemel and colleagues found on a sample of 20 individuals that a dairy-supplemented diet resulted in significant suppression of oxidative stress (plasma malondialdehyde) and lower inflammatory markers (tumour necrosis factor-alpha, interleukin, monocyte chemoattractant protein and increased adiponectin), whereas the soy exerted no significant effect. These effects were evident by day 7 of treatment and increased in magnitude at the end of the 28-d treatment period. There were no significant differences in response to treatment between overweight and obese subjects for any variable studied. They supposed that this kind of diet (rich in calcium) yields to an inhibition of calcitriol. In their ipothesis calcitriol inhibition itself decreases both adipose tissue and systemic oxidative and inflammatory stress as they demonstrated previously in obese mice (Zemel 2010).

Lasheras and colleagues assessed as conclusions of their study that dietary factors accounted for 25% of the variation in plasma MDA levels. Consumption of cooked vegetables and moderate intake of wine has been shown to be appropriate for reducing the risk of oxidative damage. On the contrary, caution must be used with the intake of potatoes because of the positive association with MDA levels. (Lasheras 2003)

Obesity

Some articles we have reviewed, investigate the relation between lipid peroxidation and obesity.

Topic	MDA =	MDA ↑	MDA ↓	TOTAL
BMI-obesity	0/4	4/4	0/4	4
BMI-obesity reduction	0/2	0/2	2/2	2
Insuline resistance	0/1	1/1	0/1	1

Table 3. *MDA and obesity in literature review.*
Note: "MDA =/ MDA ↑/MDA ↓" refers for each studied topic to the number of the most important reviewed studies where MDA levels result respectively "the same/more/less" in the study sample compared with the control group or in the same study group after the experimental intervention.

The group of Karbownik-Lewinska and the one of Sankhla demonstrated in their studies that concentration of serum malondialdehyde increased with increasing levels of Body Mass Index classification. (Karbownik-Lewinska 2012, Sankhla 2012)

Bloomer and colleagues compared in their trial blood oxidative stress biomarkers in obese (n= 14) and non-obese (n= 16) women, in response to a high fat meal. They confirmed that obese individuals have elevated resting biomarkers of oxidative stress compared to non-obese. Besides they found that the overall oxidative stress (including MDA) after meal is greater in obese women, and values appeared to remain elevated for longer periods of time post feeding.(Bloomer 2009)

Codoñer-Franch and colleagues found that oxidative stress (including MDA) increases in obese children according to the severity of insulin resistance, which could be linked to the development of comorbidities (Codoñer-Franch 2012).

Kelishady and colleagues evaluated the association of changes in oxidative and proinflammatory states with vascular function after 6-week diet and exercise intervention among obese children (n=35). They found that common inflammatory stress condition associated with childhood obesity, particularly with abdominal fat deposition, might play a role in the development of the earliest stages of proatherosclerotic inflammatory processes and subsequent vascular dysfunction and that these changes might be partially reversible by short-term diet and exercise intervention, even if patients do not reach ideal body weight. (Kelishady 2008)

Smoke and alcohol

Smoke and alcohol are other important topics of our review. We are going to face separately each subjects. The following table resume the results of our review for these two arguments.

Topic	MDA =	MDA ↑	MDA ↓	TOTAL
Smokers	2/23	21/23	0/23	23
Alcohol	1/6	5/6	0/6	6
Epatic damage from alcohol	0/1	1/1	0/1	1
Abstinence from alcohol	0/2	1/2	1/2	2

Table 4. *MDA and smoke and alcohol in literature review.*

From the biomedical point of view alcohol is a "Janus-faced" dietary component with a dose-dependent effect varying from cardiovascular protection (at low doses) to cytotoxicity. Alcohol is absorbed in the upper gastrointestinal tract by passive diffusion, is quickly distributed throughout body water and is mostly eliminated through oxidation.(Strohle 2012)

The liver is particularly susceptible to alcohol-related injury because it is the primary site of alcohol metabolism. The hepatocyte contains three main pathways for ethanol metabolism, each located in a different subcellular compartment: (1) alcohol dehydrogenase pathway of the cytosol, (2) microsomal ethanol-oxidizing system in the endoplasmic reticulum, and (3) catalase in peroxisomes (Aseervatham 2013). Each of these pathways can produce free radicals that affect the antioxidant system (Das 2006).

As alcohol is broken down in the liver, an accumulation of highly reactive molecules (free radicals and acetaldehyde) is generated. Acetaldehyde is highly toxic to the body even in low concentrations; it is also a carcinogen and a common environmental hazard. Normally, the enzyme aldehyde dehydrogenase rapidly oxidizes acetaldehyde to acetate, which travels through the bloodstream and enters other metabolic cycles that produce energy or useful molecules (Aseervatham 2013).

When consumed in moderate amounts, the major part of the ethanol is metabolized by alcohol dehydrogenase in liver (Aseervatham 2013); this is the classical pathway of ethanol metabolism (Das 2006). These processes of conversion of alcohols to aldehyde cause an increase in nicotinamide adenine dinucleotide (NADH) concentration and stimulate the conversion of xanthine dehydrogenase into its oxidase form by reducing NAD+ , which is a superoxide anion-generating enzyme that generates superoxide anions.

Free radicals generated during the metabolism of alcohols can react directly with proteins and lipids, changing their structure and functions (Aseervatham 2013). The cytocrome P450 2E1 isoform is induced by chronic ethanol consumption. Thus, this mechanism becomes more important quantitatively in the alcohol abuser. The 2E1 isoenzyme may also be a significant catalyst for formation of ROS in the alcohol consumer, as it has been demonstrated to generate high amounts of H_2O_2 (Das 2006). Also hydroxyl radicals (OH·) are generated by the microsomal ethanol-oxidizing system during the metabolism of ethanol, involving the alcohol-induced cytochrome P450 2E1 isoform. These radicals are involved in the alkylation of hepatic proteins to promote lipid peroxidation in hepatocytes by depleting glutathione (GSH) and finally leading to liver injury (Aseervatham 2013).

Peroxisomal activity also contributes to ethanol oxidation in the liver. This mechanism might be more prominent in heavy ethanol consumers where there is an accumulation of fatty acids in the liver, due to the increased peroxisomal oxidation of fatty acids (Das 2006). A common disease among the heavy drinkers is alcoholic fatty liver disease which is an early complication of heavy alcohol consumption. (Aseervatham 2013).

As previously described, ethanol oxidation gives rise to acetaldehyde, which is further oxidized by hepatic aldehyde dehydrogenases. As a result of the oxidation of ethanol by alcohol dehydrogenase and subsequent oxidation of acetaldehyde there is a significant increase in the hepatic NADH/ NAD+ ratio (Das 2006).Deposition of fat is believed to be due to an increase in NADH/NAD+ ratio followed by alcohol oxidation. Through the

electron transport chain, NADH supplies the liver with plenty of adenosine triphosphate (ATP). Due to this energy supply, other "fuels" are not essential, and consequently fatty acid catabolism is reduced. Alcoholism, which causes a fatty liver, will lead to jaundice (an indication of liver damage), enlargement of the liver, and cirrhosis.(Aseervatham 2013)

An especially dreaded clinical complication of the alcohol-induced liver disease is the hepatic encephalopathy. Its pathogenesis is a multifactor and self-perpetuating process with the swelling of astrocytes being a crucial point. Swollen astrocytes induce several reactions such as oxidative/nitrosative stress, impaired signal transduction, protein modifications and a modified gene expression profile.

The swelling of astrocytes and the change in neuronal activity are attributed to several neurotoxins, especially ammonia and aromatic amino acids. In alcohol addicted subjects multiple micronutrient deficiencies are common. The status of folic acid, thiamine, pyridoxine and zinc is especially critical (Strohle 2012) Alcohol can cause death directly by acting on the brain areas which control consciousness, respiration, and heart rate. Of all the alcohols, methyl alcohol is more toxic to humans. Methyl alcohol serves as a common adulterant in country liquor.

Generation of free radicals is enhanced in the liver during oxidation of methanol. It increases the chance of alcohol poisoning especially in developing countries (Aseervatham 2013).

The World Health Organization (WHO) has identified that consumption of alcohol is one of the top ten risks for worldwide burden of disease.

There are evidence showing that alcoholic chronic abuse of any type are a cause of various cancers of the mouth, pharynx, and larynx, oesophagus, colorectal (men), and breast. It is also probably a cause of colorectal cancer in women and of liver cancer. It increases the risk of kidney cancer (World Cancer Research Fund 2010). (Aseervatham 2013)

Thus, ethanol can affect a wide range of organ systems. Some of its effects are directly due to the action of either ethanol or its metabolites, whereas others are related to nutritional deficiencies associated with alcohol intake. Some of the tissue damage occurring in alcohol abusers are due to generation of free radicals during the metabolism of ethanol and subsequent lipid peroxidation; concentrations of antioxidants may be inadequate, especially when highly reactive oxidant species are generated in large amounts.

For this reason, several authors have attempted to study alcohol-related variations of a-tocopherol, ascorbic acid, and selenium, which are important factors in the defence against oxidative injury, and those of endogenous enzymatic antioxidants such as glutathione peroxidase (GPX) and superoxide dismutase (SOD). The results of these investigations appear to be rather contradictory, probably because of the limited size of the populations studied, and/or because of their heterogeneity with regard to alcohol consumption and the extent of liver troubles (Lecomte 1994)

The studies that we have reviewed about the linkage between MDA and the ingestion of alcohol adduce results that are mostly lined up with the biochemical alcohol properties described, even if there are some exceptions.

Lecomte and colleagues investigated a large population of 417 supposedly healthy men who consumed only low or moderate amounts of alcohol as compared with 102 alcoholic patients without severe liver disease. The 102 alcoholic patients were studied both before and after 21 days of withdrawal treatment. Plasma concentrations of antioxidant vitamins (α-tocopherol and ascorbic acid), selenium, and markers of oxidative stress, especially rnalondialdehyde (MDA) and autoantibodies directed to MDA-proteins adducts (Ig-NH2-MDA) were measured. Plasma concentrations of α-tocopherol, ascorbic acid, and selenium

were lower in alcoholics than in men who drank low amounts of alcohol, whereas MDA and Ig-NH2-MDA were higher. Plasma concentrations of α-tocophenol and selenium remained unchanged after the withdrawal period, whereas ascorbic acid, MDA, and Ig-NH2-MDA concentrations decreased. Adjustment of data for circulating lipids and nutritional intake suggests a specific effect of alcohol on antioxidant vitamins, independent of nutritional status. (Lecomte 1994).

Rolla and colleagues evaluated circulating antibodies against acetaldehyde and malondialdehyde hybrid protein adducts (MAA) in 50 patients with alcohol-induced hepatitis or cirrhosis, in 40 patients with non-alcohol-induced liver disease, in 15 heavy drinkers without liver damage and in 40 healthy controls by enzyme-linked immunosorbent assays (ELISA). The results they obtained showed the formation of acetaldehyde and malondialdehyde hybrid protein adducts antigens during alcohol-induced liver disease and suggest their possible contribution to the development of immunologic reactions associated with alcohol-related liver damage. Immunoglobulin G (IgG) reacting with the described antigens were significantly increased in the patients with alcohol-induced cirrhosis or hepatitis. The individual levels of anti-MAA IgG in those patients were associated with the severity of liver damage. Anti-MAA antibodies were also positively correlated with the levels of IgG recognizing epitopes generated by acetaldehyde and malondialdehyde (Rolla 2005).

Baldi and colleagues divided a sample of sixty-five patients with a mean alcohol intake of 151 gr/day, into four groups: alcoholics with normal liver function (7 patients), non-cirrhotic alcoholic liver disease (26 patiens) and alcoholic cirrhosis (32 patients) and the control group (15 healthy subjects) . Serum MDA was measured by the thiobarbituric acid reaction test, and mitochondrial aspartate aminotransferase (mAST) with immunochemical assay. MDA levels were significantly higher in all three groups than in controls and the highest value found in non-cirrhotic alcoholic liver disease patients (Baldi 1993)

Akkus and colleagues instead investigated plasma, erythrocyte and leukocyte lipid peroxidation, erythrocyte superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and plasma γ-glutamyl transferase (GGT) levels in 36 healthy non-drinkers aged between 18–55 years and 72 alcohol drinkers aged between 20–48 years in order to determine the oxidative effect of alcohol. Erythrocyte lipid peroxidation of the healthy controls (measured in terms of MDA) was found to be significantly reduced compared to that of the drinkers, but when using other statistic tests (tukey-HSD and F test with ANOVA) that significance disappears in those who consume less than 140 g of alcohol per day and persists in those who consume more than 140 g of alcohol per day. (Akkus 1997)

Casado and colleagues didn't find any correlation between alcohol consumption and increase of MDA circulating levels, between 50 palliative care unit workers and 50 controls. (Casado 2011)

Cigarette smoke is an other unhealthy lifestyle; it is an aerosol of complex chemical composition containing both organic and inorganic compounds such as carbon monoxide, hydrogen cyanide, and nitrogen oxides. 4800 compounds have been identified, of which 100 were known to be carcinogen, tumor initiator, and promoter.

Cigarette smoke contains two different populations of free radicals, one in the tar and one in the gas phase. Cigarette smoke radicals are formed out mainly in three ways: firstly, during combustion, both oxygen and carbon-centered radicals are expected to be produced in the cigarette flame; secondly, the formation of stable free radicals occurs in tar (between these radicals a quinine/hydroquinone (Q/QH2) radical has been identified); lastly, the oxidation of nitric oxide (NO) to the much more reactive nitrogen dioxide (NO2) happens. Molecular

oxygen is then reduced to form superoxide radicals, eventually leading to hydrogen peroxide and hydroxyl radical generation. Gas phase radicals are small alkoxyl- and carbon-centered species.

The gas phase is around 0.4–0.5 g/cigarette, containing numerous free radicals and oxidizing agents that are continuously formed and destroyed. Nitric oxide (NO·) is a species of significant importance because of its multiple physiological role such as neurotransmission and blood pressure modulator. NO· reacts quickly with molecular oxygen in air to form a toxic oxidant and nitrating agent, leading to the generation of carbon-centered radicals which subsequently react with atmospheric oxygen to form oxygen-centered radicals. Gas phase carbon-centered radicals further react with molecular oxygen to form peroxyl radical. The peroxyl radical promotes the reaction with the gas phase NO· to form alkoxyl species, and it also triggers lipid peroxidation.

The formation of peroxynitrite (ONOO−) from NO radical leads to activation of IKK (I-kB kinase) that can phosphorylate other coumpounds; the cells get inflamed by activating nuclear factor (NFkB). In addition, the principal radical in tar (quinine/hydroquinone) reacts with DNA in vitro, mostly by covalent binding, and has been shown to induce gene mutation. Synthetic Q/QH2 polymers have been shown to be potent redox catalysts in organic chemistry and may also have the capability of altering oxy-radical levels in the lungs.

Apart from free radicals, there are some polycyclic aromatic hydrocarbons. present in the tobacco smoke.

One such hydrocarbons is benzo(a)pyrene diol epoxide, which is a strong carcinogen.. In passive smoking, the environmental air contains nicotine, benzo(a)pyrene, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, and quinine. Benzo(a)pyrene is metabolically activated into benzo(a)pyrene diolepoxide by reacting with N2 position of quinine to produce N2 guanine lesions (BPDE-N2-dG), which is highly unstable.

The BPDE-dG adduct formed, can accumulate in human tissues, especially in the bronchial cells, thus leading to the formation of lung carcinoma. Nicotine was first prescribed as a medical drug to treat rodent ulcer and constipation; instead it is the major additive component of cigarette that harms our body system.

The physical effect of tobacco smoke on the oral tissues by heat and/or the direct effect of nicotine stimulates melanocytes that are located along the basal cells of the epithelium to produce more melanosomes. This condition results in the increased deposition of melanin in heavy smokers. Melanins are naturally occurring polymers containing quinone and hydroquinone groups that are derived from tyrosine via oxidation to dihydroxyphenylalanine. By adding metal ions (e.g., Cu^{+2}), the oxidation process is accelerated via Fenton cycling. In this oxidation process, NADH is the ultimate source of reducing equivalents from melanin quinone groups to the semiquinone ones which then reduce dioxygen to superoxide. The ultimate product, hydrogen peroxide, could then be formed either by dismutation of superoxide or by the oxidation of another NADH molecule by superoxide. Melanins also have been shown to catalyze the reduction of dioxygen to hydrogen peroxide in the dark. Each puff of smoke contains over ten trillion free radicals, which results in tumor due to initiation as well as repeated attacks from ROS on cellular macromolecules.

It has been shown that the nicotine and tar levels produced by burning cigarettes are three times higher than in the smoke directly exhaled by the smokers and the concentration of carbon monoxide is approximately five times higher. They also proved that passive smoking is tied with various adverse effects on babies such as increased risk of sudden death syndrome, asthma, chronic diseases in the middle ear, slow lung growth, etc.(Aseervathan 2013)

The studies revised in the literature about the linkage between MDA and smoke, underline the correlation between the increase of MDA levels and smoke. (Peluso 2012, Kurtul 2012, Li 2007, Bloomer 2007, Venkatesan 2006, Solak 2005, Celec 2005, Lykkesfeldt 2004, Mircea 2004, Vassalle 2002, Ozbay 2002, Aluntas 2002, Kharb 2001, Codandabany 2000, Miller 1997, Casado 2011, Casado 2008, Nielsen 1997).

Moreover Zawadzki and colleagues observed that effects of passive exposure to tobacco are similar to those of active smoking. They enrolled a total of 104 students, exposed to passive smoking. The subjects were categorized in three subgroups depending on nicotine metabolite levels in blood (subgroup I with metabolite level >100 ng/ml (high exposure); subgroup II with the metabolite level of 10–100 ng/ml; subgroup III with metabolite level <10 ng/ml). The results showed statistically significant differences in levels of lipid peroxidation metabolites and catalase (CAT) activity. Levels of MDA and of 4-HNE (4-idroxynonenal) were higher in subgroup with nicotine metabolite level >100 ng/ml (high exposure) than others. (Zawadszki 2008)

The study conducted by Tonguc and collegues yields to the conclusion that systemic and local MDA levels are increased by peridontitis in addition to the increase that smoke itself causes. (Tonguc 2011).

Also Guentsch and colleagues had arrived to the same conclusion. They compared thirty healthy subjects (including 15 smokers) to thirty periodontitis patients (including 15 smokers). Malondialdehyde, glutathione peroxidase and the total antioxidant capacity were recorded in saliva. The lowest level of lipid peroxidation (MDA) was measured in saliva in the non-smoking periodontally healthy subjects. MDA levels were significantly higher in periodontitis patients compared to non-smoking controls. Non-surgical periodontal treatment leads to a reduction of MDA and glutathione peroxidase levels comparable to healthy controls. (Guentsch 2008)

Bamonti conducted an interesting pilot study. 32 healthy volunteers, 16 light smokers and 16 non-smokers, on twice daily supplementation of mixed fruit and vegetable juice powder concentrate were monitored at time zero and after 30 days. Baseline free malondialdehyde concentrations were significantly higher in smokers than in non-smokers and normalised after 30-day supplementation. (Bamonti 2006).

We picked out also few studies that yields results contrasting to the previous studies.
Marangon and collegues assessed the association between smoking, food consumption, and antioxidant vitamin intake and plasma indexes of oxidative stress and antioxidant defences in 459 French adults healthy men aged 23-57. Smokers ate less fruit and vegetables than nonsmokers, leading to lower vitamin E, vitamin C, and carotene intakes, even after adjustment for age, education, and marital status. Unlike vitamin E, plasma ascorbic acid and beta-carotene concentrations were reduced in smokers compared to nonsmokers and were inversely related to cigarette consumption. This difference remained significant after adjustment for alcohol and dietary intakes. Among the measured oxidative stress indexes, only Schiff base concentration (and not MDA) was positively related to the number of cigarettes smoked. MDA levels instead did not show any correlation with smoke. (Marangon 1998)

Also Ayaori and colleagues didn't find significant differences in plasma levels of thiobarbiturate reactive substance (TBARS), and the levels of lipid peroxides (LPO) between the study population they analyzed. consisted of 149 healthy males: 75 active smokers (consumption of > 15 cigarettes/day for more than 5 years), 36 passive smokers (more than 10 hours/week exposure to environmental cigarette smoke), and 38 nonsmokers (no cigarette smoke exposure). (Ayaori 2000)

Ermis and colleagues didn't note any significant differences among the study groups (14 active-smoking, 14 passive-smoking, and 15 nonsmoking mothers and their newborns on day 7 post-partum) regarding MDA or superosside dismutase (SOD) levels. (Ermis 2004).

Physical exercise

Regarding physical exercise, there are many complex elements that should be considered. Oxidative stress can result from numerous factors, including excess food intake, body fatness but also extremes of physical activity. Sedentary living specifically increases the risk of diseases associated with hypokinesis and obesity The point is that exercise reduces the clinical diseases risk, but may promote the production of free radicals.(Alessio 2000)

The well-documented benefits of regular physical exercise include a reduced risk of cardiovascular disease, cancer, and osteoporosis. The complex mechanisms that contribute to these effects include decreased adipose tissue, altered lipid and hormonal profiles, receptor and transport protein adaptations, improved mitochondrial coupling, and enhancement of antioxidant defenses. (Aseervatham 2013)

Aerobic organisms produce ROS during normal respiration and so do stress conditions. (Aseervatham 2013)

Physical activity increases the generation of free radicals in several ways.(Urso 2003).
Particularly eccentric exercise involves high force during the lengthening portion of muscle contraction. This can occur involuntarily or voluntarily during conditions in which the activated muscle cannot produce enough force to overcome the resistive force (for example, during heavy resistance training) or during an intentional production of submaximal force in order to control the eccentric (lengthening) movement (for example, controlled lowering of external load and/or downhill running). This can create an imbalance between oxidant and antioxidant levels, leading to oxidative stress. Primary ROS generation in response to acute exercise can occur via several pathways.
These include mitochondrial respiration (electron leakage from electron transport chain and subsequent production of the superoxide radical), prostanoid metabolism, the auto-oxidation of catecholamine, and oxidase enzymatic activity (NAD(P)H oxidase, xanthine oxidase).

Although the main function of mitochondria is energy production, they generate reactive oxygen species during oxidative phosphorylation.
Release of such intermediates accounts for an estimated 1 to 5% of the oxygen consumed during respiration, depending on the substrate and respiration state. Skeletal muscle can increase its oxygen consumption up to 20-fold between rest and exercise; conseguently also oxidative phosforilation increase and this could be an important mechanism for controlling reactive intermediate production.
Xanthine dehydrogenase is present in endothelial cells of blood vessels and skeletal and cardiac muscles. It converts NAD to NADH. Oxidation of purines to uric acid is catalyzed by xanthine oxidase which is present in serum (Aseervatham 2013). Xanthine oxidase, NAD(P)H oxidase are the enzyme particularly involved in radical formation (Urso 2003)

During exhaustive physical exercise, increased metabolic activity consequently increases the expenditure of ATP. Thereby, it enhances the activation of free radical-generating enzymes like NADPH oxidase and xanthine oxidase. As a result of catabolism, ATP hypoxanthine is produced, and H_2O_2 is generated. By Fenton's reaction, it again undergoes reduction to form hydroxyl radical (HO.) and dioxygen (O_2-), which can induce lipid peroxidation and DNA damage.When exercise period finishes or deacreases in intensity, more blood arrives to muscle than during exercise (reperfusion).

During reperfusion blood yields to the muscle more oxygen than during the previous exercise condition. Oxygen is reintroduced, xanthine oxidase produces radicals as byproducts, contributing to overall oxidant and free radical formation.This mechanisms are particularly involved during anaerobic exercise. (Aseervatham 2013). Also catecholamines that are released during exercise can lead to free radical production. (Urso 2003),

Phagocytic white blood cells also produce potent oxidants, generating reactive species that kill invading pathogens. However, neutrophils can infiltrate damaged skeletal muscle following strenuous or eccentric exercise, further damaging the cells. White blood cells also carries xanthine oxidase, which leads to the conversion of hydrogen peroxide in the presence of water and oxygen. Relaxation techniques and breathing exercises can help to reduce and withstand stress better, and also help to relax facial muscles and skin. (Aseervatham 2013)

Topic	MDA =	MDA ↑	MDA ↓	TOTAL
Diet and physical activity	0/2	0/2	2/2	2
Frequent physical activity	0/5	3/5	2/5	5
Moderate aerobic physical activity	1/2	0/2	1/2	2
Strong physical activity	0/2	2/2	0/2	2
No physical activity	0/2	1/2	1/2	2

Table 5. *MDA and physical exercise in literature review.*
Note: "MDA =/ MDA ↑/MDA ↓" refers for each studied topic to the number of the most important reviewed studies where MDA levels result respectively "the same/more/less" in the study sample compared with the control group or in the same study group after the experimental intervention.

With attention to aerobic exercise, it has been demonstrated cardiovascular and metabolic benefits such as increased maximal oxygen consumption, improved aerobic endurance capacity, and increased energy production via the mitochondria respiration system. The production of either reactive oxygen species occurs in an intensity and duration dependent manner. Some authors speculate that low degree of radical production should be viewed as beneficial rather than detrimental, serving as a stimulus to upregulate key endogenous anti-oxidant protective mechanisms (Leelarunngrayub 2011).

The studies that we have reviewed about the linkage between MDA and physical activity adduce results that are mostly lined up with the biochemical smoke characteristics described.

Mergener and colleagues analyzed 110 females, aged 66.3+/-8 years classifying them into sedentary (n=54), walking (n=36) and muscle building (n=20) groups. MDA levels were significantly higher and GPx levels were significantly lower in active groups than in sedentary group. They suggest the existence of undiscovered biochemical pattern that could be responsible for health benefits, despite radical production, particularly for older adults that do exercise regularly (Mergener 2009).

Franzoni and colleagues studied the relationship between long-term physical activity, plasma antioxidant status, and artery endothelial function in young and older healthy men. Their results suggested that regular physical activity is associated with preserved antioxidant defenses and endothelial function in older individuals.
Regarding plasma malondialdehyde and antioxidant capacity (as total oxyradical scavenging capacity), they evaluated young (n = 16) and older athletes (n = 16) and matched healthy

sedentary subjects (n=34). Sedentary older subjects showed higher MDA levels and lower plasma antioxidant capacity as compared with the other subgroups, whereas in older athletes MDA levels and antioxidant capacity were similar to those observed in the young subgroups (Franzoni 2005).

Cardoso and colleagues examinated thirty-four women, selected and divided into three groups (resistance exercise group (N = 12, performing 60 min of resistance exercises), spinning group (N = 12, performing 60 min of spinning, and control group (not exercising regularly, N = 10)), measuring blood lymphocytes and monocytes, MDA and antioxidant activities one hour after exercise.
They demonstrated that an acute boust of intermittent or anaerobic exercise induces immune suppression and increases the production of reactive oxygen species, causing oxidative stress in middle-aged and trained women. Furthermore, trained women showed improved antioxidant capacity and lower oxidative damage than sedentary ones, demonstrating the benefits of chronic regular physical activity (Cardoso 2012).

Similarly, Munoz Marin and colleagues demonstrated in a study conducted on twenty male trained cyclists, that short time of high intensity cycling leads to oxidative stress increasing plasma (including MDA) and decreasing erythrocyte vitamin C levels. (Munoz Marin 2010)

El Abed and colleagues enrolled 10 competitive judokas and 10 sedentary subjects after mixed exercise (anaerobic followed by aerobic). Blood samples were taken immediately after the physical exercise (P0), and at 5 (P5), 10 (P10), and 20 (P20) minutes post exercise. They evaluated antioxidant enzymes superoxide dismutase, glutathione peroxidase, and glutathione reductase, in addition to α-tocopherol, and total antioxidant status, and malondialdehyde (as a representation of lipid peroxidation).
They observed that competitive judo athletes have higher endogenous antioxidant protection compared to sedentary subjects and that both groups experience a similar increase in exercise-induced oxidative stress (measured as MDA) (El Abed 2011).

Caimi and colleagues arrived to similar conclusion in their study involving 81 unprofessional athletes subdivided into three subgroups. The first group included 28 subjects who practiced endurance sports, the second included 30 subjects who practiced mixed sports, the third included 23 subjects who practiced power sports. 61 sedentary persons were enrolled as controls. Lipid peroxidation, measured as TBARS, were increased and the total antioxidant status decreased, in the whole group of athletes in comparison to the sedentary controls.(Caimi 2011).

Yadav and colleagues demonstrated that yoga reduces oxidative stress. The serum concentration of TBARS decreased significantly in the blood of the 104 enrolled subjects after ten days of yoga programme practice. (Yadav 2005).

The study of Karbownik-Lewinska and colleagues evaluated the change in blood oxidative stress, blood interleukin-2, and physical performance following 6 weeks of moderate intensity and duration aerobic dance exercise in 24 sedentary women. Blood samples were collected at rest twice before (baseline) and after the 6-week intervention, for analysis of protein hydroperoxide, malondialdehyde, total anti-oxidant capacity (TAC), and interleukin-2 levels.
They concluded that aerobic dance exercise at a moderate intensity and duration can improve physical fitness, decrease MDA, and increase TAC and IL-2 in previously sedentary women. (Karbownik-Lewinska 2012)

Environmental factors and occupational exposure

Regarding environmental stress, it can increase the production of reactive oxygen and nitrogen species (RONS), decrease the antioxidant production, causing metabolic changes in animals, plants, and humans. Occupational exposure to metals, benzene, cement dust, and multiple other agents is linked with increased lipid peroxidation, increased DNA oxidation, and decreased levels of vitamin E and C; it leads to diseases such as neurotoxicity, cancer, liver damage, cardiovascular diseases, inflammation, respiratory diseases as well as through changes in gene expression that promote apoptosis when needed, and systemic inflammation. Exogenous pollutants generating free radicals are part of our daily inhaling/ingesting life, and there is no protection or escape from them.

Many environmental factors and chemical pollutants induce the generation of ROS. Air pollution is the most harmful form of pollution in our environment. Air pollution is composed of a diverse mixture of particulate matter, gases (for example, ground-level ozone, carbon monoxide, sulfur oxides, nitrogen oxides), organic compounds (for example, polycyclic aromatic hydrocarbons and endotoxins), and metals (vanadium, nickel, and manganese) present in outdoor and indoor air. Of these components, particular matter and ground-level ozone are the most widespread health threats and have been implicated in various diseases.

These free radicals can accumulate in air from burning fuels in smokestacks, car exhaust pipes, or house chimneys or from the formation of ground-level ozone during hot weather. Free radicals in air pollution may be 300 times as damaging as those from tobacco smoke.

Some of these radicals are associated with increased immune response in some respiratory diseases, creating worse symptoms. It has been suggested that the nitrate radical and sulfur dioxide could be the main responsible for the respiratory diseases. It is reported that the atmospheric nitrate radical irreversibly damages amino acids which are the building blocks for protein in the human body. In addition, it could potentially cause damage to peptides lining the respiratory tract and may contribute to pollution-derived diseases.

The nitrate radical is formed from many sources; one common source is nitrogen dioxide which itself is emitted in the environment from car exhaust. The nitrate radical reacts with amino acid to form compounds such as beta-nitrate esters, beta-carbonyl, and aromatic nitro compounds

While it is well known that air pollution affects human health through cardiovascular and respiratory morbidity and mortality, it has recently been shown that these deleterious effects extend to the brain. Ultrafine (nanosized particles) and fine particles are the most notorious air pollution components, penetrating lung tissue compartments that reach the capillaries and circulating cells (for example erythrocytes). Experimentally, inhalation or nasal instillation of ultrafine particles in rodents results in the translocation of the particles into the systemic circulation. Microglia is also reported to respond to titanium nanoparticles by producing ROS, which are neurotoxins. Partcicular attention should be used regarding heavy metals.

Despite the toxic and carcinogenic effects of metals in human and animals that have been reported in many studies, it is also well known that these metals form a crucial part in normal biological functioning of cells. Anthropogenic activities such as mining, combustion of fossil fuels, application of phosphates and sewages for agricultural production and industrial manufacturing, leads to the accumulation of heavy metals in the environment.

The heavy metals such as lead, mercury, and cadmium have electron sharing affinities that can result in the formation of covalent attachments mainly between heavy metal and

sulfhydryl groups of proteins, subsequently generating reactive species, which in turn may cause neurotoxicity, hepatotoxicity, and nephrotoxicity in humans and animals.

Furthermore it has been reported that certain heavy metals (arsenic trioxide, chromium, and vanadium) stimulate inflammatory pathways such as the NF-kB cascade, resulting in chronic inflammation diseases and oxidative damage.

Many data provide evidence that metals like chromium, nickel, and manganese are capable of interacting with nuclear proteins and DNA, causing oxidative deterioration of biological macromolecules which results in cellular damage like depletion of enzyme activities and controlling metabolic pathways (Aseervatham 2013).

Topic	MDA =	MDA ↑	MDA ↓	TOTAL
Stress from work	0/3	3/3	0/3	3
Environmental and industrial pollution	0/3	3/3	0/3	3
Married people	0/1	1/1	0/1	1

Table 6. *MDA and"enviromental factors and occupational exposure" review.*
Note: "MDA =/ MDA ↑/MDA ↓" refers for each studied topic to the number of the most important reviewed studies where MDA levels result respectively "the same/more/less" in the study sample compared with the control group or in the same study group after the experimental intervention.

About the linkage between environmental pollution and MDA increasing levels, there are evidence emerging from the studies of Peluso and colleagues.

In 2012 they conducted a cross-sectional study to compare the prevalence of malondialdehyde-dG adducts in groups of 173 subjects living near industrial point emissions of Map Ta Phut Industrial Estate in Thailand.
The sample population was divided in groups (petrochemical workers, nearby residents, and subjects living in a control district without proximity to industrial sources) according various degrees of air pollution experienced. The multivariate regression analysis showed that the adduct levels were associated with occupational and environmental exposures to air pollution. The highest adduct level was observed in the steel factory workers (Peluso 2012).

In 2012 Peluso and colleagues conducted another study on living near industrial point emissions of Map Ta Phut Industrial Estate in Thailand.
The aim of the study was another because they wanted to evaluate the influence of fruit and vegetables, and fried and charcoal-grilled/barbecued food consumption on M1dG. It is interesting that no association of diet with M1dG was found in industrial estate workers. It is reasonable to assume that the antioxidant properties of a diet rich in fruit and vegetables was contrasted by the genotoxic effects of heavy air pollution exposure.(Peluso 2012)

Another study about this topic was conducted by Babu and colleagues. They found a significant increase of plasma lipid peroxidation and a significant decrease of superoxide dismutase and glutathione peroxidase levels in the study group (workers exposed to Cadmium during electroplating) compared with the control group (Babu 2006).

Georgieva and colleagues found an elevation of MDA levels and decrease of glutathione perossidase and glutatione in workers (petrochemical) exposed to a mixture of hydrocarbons. The decreased values of MDA and the increased gluthatione peroxidase activity and gluthatione concentrations at the second examination proved the positive effect

of the treatment with tablets containing vitamins, micro-elements, and bioflavonoids, in appropriate doses (Georgieva 2002).

Not only environmental stressors, but also lifestyle stress generates an increase of lipid peroxidation and of MDA circulating levels.

Casado and colleagues determine superoxide dismutase (SOD) and catalase (CAT) activity, and malondialdehyde (MDA) levels in nurses of a hospital intensive care unit. The sample surveyed was composed by thirty-two nurses working in an intensive care unit and 35 aged-matched healthy individuals of both sexes as a control group. MDA levels increased with age in both nurses and control group; MDA levels, were lower in those who practice frequently sports and have a diet rich in fruit and vegetable.
Significant differences in MDA levels were found between the nurses sample (higher MDA levels) and the control group for all age groups meaning that occupational stress can increase free radical generation and MDA levels. Moreover higher SOD activity and MDA levels were detected in nurses on evening and night shifts. Significant variations in MDA levels were also detected between single (lower MDA levels) and married (higher MDA levels) people. (Casado 2008)

In the years later, Casado and colleagues conducted another study about the same topic. This time, they surveyed fifty-two palliative care unit workers and 50 gender and aged matched healthy individuals as controls. MDA concentrations resulted increasing with age in controls and palliative care unit workers, and significant differences in MDA between controls and palliative care unit workers for all age groups were noted, confirming that occupational stress increases generation of reactive oxygen species and oxidative stress levels.
Besides SOD activity and MDA concentrations resulted higher in palliative care workers who work the evening and night shifts, and these workers also showed significantly higher levels of stress. Significant variation in MDA concentrations were also detected between unmarried (lower levels) and married individuals (higher levels), but any variation was found with respect to divorced individuals. Moreover lower MDA levels were found for those who have a diet rich in fruit and vegetable, for those who practice regularly some kind of sports while higher MDA levels were detected in smokers. (Casado 2011)

Tsuboi and colleagues analyzed a sample female nurses working in a university hospital (n=18 with high job stress and n=15 with low job stress) and measured cholesterols, lipid peroxidation (MDA) and antioxidants in the plasma.
They concluded that psychological stress may reduce the plasma levels of LDL+VLDL accompanying an alpha tocopherol decrease. Moreover they found a correlation between elevated MDA and depressive symptoms in low job stress participants (in the high stress group this correlation did not result statistically significative) (Tsuboi 2007).

MDA and cancer

First evidences implicating a linkage between lipid-derived toxicants and tumour-genesis has been found many years ago. Associations between the intake of dietary fat and cancer incidence or mortality at several tissue sites were first clarified by epidemiologists - Armstrong and Doll, 1975, Doll, 1972- several decades ago. In addition, early animal studies-Tannenbaum, 1942; Carroll, 1991- suggested that the growth of tumours could be increased by adding PUFAs to the diet of rodents. In other experiments-Gammal 1967, Roebuck 1981-, dietary supplementation with PUFAs increased the incidence of mammary and pancreatic cancer in animals 'initiated' with xenobiotic carcinogens (Burcham 1998)

Esterbauer reported some studies addressing carcinogenicity of orally administered oxidized oils. In one –Nolen 1967-, no increase of cancer frequency in mice was found, whereas another study-Tinsley 1981-, found an increase in mammary tumors in mice. Several studies-Bird 1982, Spalding 1989, Siu 1983, Shaumberg 1974- found that chronic oral administration of MDA to mice in the drinking water or application to skin, followed by a tumor promoter, increases tumor frequency in mice. Some authors-Bull 1988, Earles 1991- hypothesized that lipid hydroperoxides could have a tumor-promoting effect stimulating cell proliferation in the colon (Esterbauer 1993).

The demonstration-Brambilla 1989, Cao 1995- that radioactivity becomes irreversibly associated with DNA after adding radiolabelled PUFAs or lipid hydroperoxides in cultured cells, confirmed that genotoxic substances are formed from lipids). Carter and collegues found an indirect evidence of cancer linkage to lipid peroxidation, demonstrating that coadministration of cyclooxygenase inhibitors often descreased tumour area in experiments and thus suggesting a tumourogenic role of lipid peroxidation products formed in prostaglandins pathway (Burcham 1998)

The involvement of lipid peroxidation in cancer aetiology is probably the clearest example of linkage between MDA and pathology as reviewed by Del Rio in 2005.(Del Rio 2005)

Lipid peroxidation begins by free-radical attack of membrane lipids, generating large amounts of reactive products, which are implicated in tumor initiation and promotion. Modification of DNA is demonstrated to be an important early step in carcinogenesis and endogenous DNA adducts, derived from oxidative stress or lipid peroxidation or other endogenous processes, are considered important contributors to the etiology of human cancer (Leuratti 2002).

Tumor cell proliferation needs rapid synthesis of macromolecules including lipids, proteins, and nucleotides. Cancer cells undergo metabolic adaptation as a result of the expression of oncogenes such as Ras, Src or Bcl-Abl, which causes changes in genes expression of particular enzymes (hexokinase2, TKTL1, PDK), thus altering the mitochondrial activity. This process is mostly started by the hypoxia inducible factor (HIF). A consequence of HIF activation is an increase in glucose uptake and phosphorylation due to elevated levels of both the glucose transporter Glut-1 and the glucose phosphorylating enzyme hexokinase. Warburg observed that proliferating tumor cells use glucose at a high rate and release as final products lactate instead of CO_2, performing in this way an anaerobic glycolysis. Infact the high glycolytic flux needs nicotinamide adenine (NAD^+), which is efficiently regenerated from the conversion of pyruvate into lactate in the anaerobic glycolysis. This metabolic conversion makes glycolysis self-sufficient as long as high glucose uptake is possible, also in anaerobic conditions. Anyway the tricarboxylic acid (TCA) cycle is active and characterized by an efflux of substrates which are used in other biosynthetic pathways, particularly fatty acid synthesis. The success of this synthetic activity depends on the

activation of pathways that generate reductive power (NADPH) and restore oxaloacetate for continued TCA cycle function (anaplerosis). Both these needs are met by the high rate of glutamine metabolism that transformed cells have. The end-product of glutaminolysis is, as for glycolysis, lactate, which is a product of pyruvate oxidation by LDH. Also, glutamine is critical to maintain the pool of glutathione, which is important for antioxidant defenses. The gamma glutamyl cycle, apart from explaining the synthesis and degradation of glutathione, can generate signals that promote active amino acid transport into cells Therefore, enzymes that catalyze reactions involved in glycolytic metabolism, glutaminolysis, and the gamma glutamyl cycle are important tools for monitoring and treating cancer.

Increase of reactive oxygen species (ROS) leads to a growth in lipid peroxidation, whose products act as carcinogenetic, irreversibly damaging DNA. The presence of ROS determine a cascade of reaction that may change, step by step, a normal cell into a cancer one.

It has been demonstrated that most types of cancer cells have increased levels of (ROS).

Anyway a mild increase in ROS can promote cell proliferation and differentiation whereas high amounts of ROS can cause oxidative damage to lipids, proteins, and DNA.
ROS levels are higher in cancer cells, for the following reasons. The expression of genes linked with tumor transformation, such as Ras, Bcr-Abl and c-Myc, induce ROS production. In addition to oncogenic transformation, mitochondrial DNA (mtDNA) mutations have also been detected and correlated to increased ROS levels in certain types of cancer cells, including those in solid tumors and leukemia. Several protein components of the electron transport chain are encoded by mtDNA. Thus, mutations of mtDNA are likely to cause impairments in electron transfer, leading to leakage of electrons and the generation of superoxide, which can subsequently produce other types of ROS.
A so high increase in ROS stress can induce oxidative damage of cellular components leading to cell death; instead cancer cells are equipped with sufficient adaptive mechanisms to tolerate the ROS stress and are able to survive to these stress conditions. The adaptive processes involve activation of certain redox-sensitive transcription factors, which consequently lead to increased expression of the downstream genes encoding various ROS-scavenging enzymes and redox-sensitive survival machineries. Thus, increased ROS stress in cancer cells is likely to increase expression of SOD and other antioxidant enzymes. (Farias 2011)

Anyway free radicals are intense toxicant because of their being very active in the biochemical nature and oxidizing ability (Wang 1996) and through lipid peroxidation and other pathways can provoke further damages to DNA, making cancer cells stronger to chemotherapies.

During the years several studies on humans and several laboratory experiments on cultured cells and animals have been carried on by numerous authors, studying different kind of cancers and their linkage to lipid peroxidation.
They mostly agree that, in presence of a cancer, MDA levels increase.

We have reviewed the literature about the correlation between MDA and colon, breast and gastric cancer until June 2013. We used Pubmed (www.nbci.nml.nih.gov/pubmed) as research engine. We verified various research key. We focused on case-control studies conducted on humans, excluding those on animals, and on laboratory studies where sample of neoplastic tissues are compared to normal ones.

Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer in both men and women and the second most common cause of cancer death.(Farias 2011)

Despite a broad range of treatments, up to 50% of patients will inevitably develop incurable metastatic disease.(CaiFang 2012)

Appropriate staging is essential for choosing the proper therapy for a patient; the tumor node metastasis (TNM) system (maintained collaboratively by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC)) is the most clinically used staging system. (Farias 2011)

Approximately 94% of CRC is sporadic in nature, and it is considered that 75–80% might be imputable to environmental causes. CRC develops through a multistep sequence of dysplastic morphological change, characterized by an accumulation of genetic defects. This sequence most commonly includes an adenoma. The risk of developing cancer grows with the number and size of adenomas and villous histology.

Diet is estimated to have the most important environmental influence on CRC. High assumption of red meat has found associated with increased risk of CRC. Also obesity, low rate of physical activity and alcohol chronic abuse are correlated to an increased risk of CRC. Instead high consumption of vegetables, fruit and fibers has been shown to reduce the risk. In addition, it has been reported that subjects who maintained high levels of physical activity throughout their lives are at lower risk for developing colon cancer (Leuratti 2002).

Thus, colorectal tissue is constantly exposed to a variety of potentially dangerous chemicals, such as drugs, food additives and food constituents, that may be act as carcinogens. Free radicals are formed during the metabolic activation of these compounds and this have been reported to be an important factor in carcinogenesis (Ozdemirler 1998).

Lipid peroxidation begins by free-radical attack of membrane lipids, generating large amounts of reactive products, which are implicated in tumor initiation and promotion. (Leuratti 2002).

Morover, an increasing number of studies assert that colorectal cancer is associated with high levels of lipid peroxidation, mainly due to neoplastic tissue metabolism. Infact lipid peroxidation happens when ROS are produced close to or within membranes and attack the fatty acid side chains of membrane phospholipids. Similar to the increase of ROS, lipid peroxidation increases during the course of carcinogenesis in colorectal cancer (Farias 2011) Increased levels of MDA, together with increased levels of PUFAs and prostaglandins PGE2, have been reported in tumor tissues of CRC patients as compared with their normal mucosa (Leuratti 2002).

Our review of the literature support the association between colon cancer and increase of MDA levels as shown in detail in next tables.

Author	Journal	Year	Country	Population	MDA	Increase of MDA levels (cancer pts versus controls)
Bhagat SS	Indian J Physiol Pharmacol	2011	India	K: 24 Control group: 24	Serum concentration	x
Farias I.L.G.	Biomed Farmacother	2011	Brazil	K:43 Control group: 20	Serum concentration (TBARS)	x
Chandramathi S.	J.Cancer Research and Clinical Oncology	2008	India	K: 49 Control group: 95	Urinary concentration	x
Chang D.	Biomed Envirom Sci	2008	China	K: 36 Control group: 40	Serum concentration	Reduction of MDA levels in K versus controls; increase of other index of oxidative stress in K versus controls
Nayak BS	Scand J Gastroenterol	2007	India	K: 30 Control group: 30	Plasmatic concentration	x
Regoly-Merei A.	Orv Hetil	2007	Hungary	-	Plasmatic concentration	x
Updadhya S.	Indian Journal of Clinical Biochemestry	2004	India	K: 17 (Dukes stage B) Control group: 20	Plasmatic concentration	Reduction of MDA levels in K versus controls
Lauschke H.	Eur Surg Res	2002	Germany	K: 36 Control group: -	Serum concentration	x
Hietanen E.	Eur J Clin Nutr	1994	Finland	-	Serum concentration	-

Table 7. *MDA and colorectal cancer (case-control studies conducted on humans, literature review)*
The table summarizes the case-controls studies selected according to our review strategy. Full text was not available for the articles of Regoly-Merei 2007, Hietanen 1994 and Lauschke 2002. For this reason some information are lacking.

An another interesting study was conducted by Leung and colleagues. They compared a sample of 53 patients with operable CRC cancer to a sample of 53 patients with not-operable CRC cancer. They found plasmatic concentration of MDA (measured as TBARS) lower in the sample of patients with operable CRC tumour than in the other group. The sample of patients with not-operable CRC cancer presented also lower levels of circulating antioxidants. We decided to reserve to this study a special paragraph, because the study has been conducted comparing two groups of persons with different colon cancer stage. It is interesting that higher levels of MDA have been detected in the group of patients with higher cancer stage The study has been carried on in UK and it has been published in 2008 in the International Journal of Cancer (Leung 2008)

Author	Journal	Year	Country	Sample KT*/NT§	MDA	Increase of MDA in sample with cancer
Kekec Y.	European Journal of International Medicine	2009	Turkey	33/33	Tissues concentration	x
Murawaki Y	Cancer Lett	2007	Japan	43/43	Tissues concentration	x
Rainis T	Digestive Diseases and Science	2006	Israel	25 /25	Tissues concentration	x
Skrzydlewska E.	World J Gastroenterol	2005	Poland	81/81	Tissues concentration	x
Ozdemirler Eratal G.	JPN J Clin Oncol	2005	Turkey	20/20	Tissues concentration, after centrifugation (TBARS, spectrophotometry)	x
Skrzydlewska E.	J of Toxicology and Enviromental Health	2011	Poland	55/55	Tissues concentration	x
Ozedemirler G.	J Cancer Res clin Oncol	1998	Turkey	10 /10	Tissues concentration	Absence of significant results
Hendriickse CW	Br J Surg	1994	UK	20/20	Tissues concentration	x
Hietanen E.	Eur J Clin Nutr	1994	Finland	-	Tissues concentration	-
Otamiri T	Cancer	1989	Sweden	-	.	x

Table 8. *MDA and colorectal cancer (laboratory studies where sample of neoplastic tissues are compared to normal ones, literature review)*

KT*: sample of neoplastic tissue; NT§: sample of normal adjacent tissue

The table summarizes the laboratory studies where sample of neoplastic tissues are compared to normal ones,selected according to our review strategy. Full text was not available for the articles of Hietanen 1994 and Otamiri 1989. For this reason some information are lacking.

Also other interesting experiments study the correlation between MDA and colon cancer, even if from different points of view. We report the most significant ones.

Ozdemirler and collegues demonstrated the decreased production of HPS70 in colon rectal cancer tissues compared to normal colon mucosal samples, and the concomitant inverse increase of MDA levels. The heat shock protein act as "chaperonine" and are constitutively produced by our cells. They hold in their molecular conformation the new synthesized proteins to help in the correct allocation of them. In stress conditions the production of heat shock proteins is increased, because they are implicated in the individuation and destruction of damaged or misfolded proteins. HPS70 is the most studied heat shock protein (Ozdemirler 2005).

Some authors found higher levels of Cox 2 in colorectal cancer and adenomas compared to normal tissues. High levels of Cox-2 are representative of high levels of inflammation and they correlate with higher levels of prostaglandins and consequently with higher levels of prostaglandins metabolism products, such as MDA. (Wendum 2004, Hendrickse 1994) Thus, it is obvious that dietetic supplementation of salicylic acid, that lead to a decrease in phlogosis and prostaglandins levels, yields also to a reduction of MDA levels (Drew 2005).

With regards to antioxidants, in colon cancer tissues compared to normal ones or in subjects with colon cancer compared to healthy ones, different data are available in the literature reviewed. Some authors found also discordant levels of different antioxidants in the same sample or population. (Updadhya 2004, Murawaki 2007, Kekec 2009, Rainis 2006, Skrzydlewska 2001, Skrzydlewska 2005, Leung 2008, Bhagat 2011).

As reported in the previous paragraph, the assumption of lipid with diet in healthy population, leads to an increase in lipid peroxidation and in MDA levels. Instead many authors demonstrated with laboratory experiments that the administration of fatty acids to cancer cell line population leads to a inhibition of the growth of neoplastic cells, even if there is an enhancement in lipid peroxidation (Paolazza 1999, Nano 2003, Xiaofeng Lu 2010,Tsuzuki 2004) and to a reduction of SPRC (a glycoprotein which endues to cells, also cancer ones, the property of sticking to extracellular matrix) (Watkins 2005)

An explanation for this results, is that MDA and other lipid peroxidation products, can have cytotoxic effect in normal cells and also in CRC cancer cells, which implies a potential role of PUFAs in CRC treatment (Cai Fang 2012); moreover lipid peroxidation and its products can damage mitochondrial membrane, partly blocking cells proliferation (Xiaofeng 2010). Another possible explanation for the few negative results of MDA elevation in cancer patients can be found in TGF1β, which helps cancer growth and progression, and which results decreased with decreasing levels of lipid peroxidation products (Biasi 2002).

Morover it is interesting that malondialdheyde levels have been detected diminished by some authors after different therapeutic interventions (surgery-Surinenaite 2009, Lauscheke 2002), even if Updaya and collegues didn't confirm these results.(Updadhya 2004)

Lauscheke and collegues found an increase of MDA levels parallel to the worsening of T stage in colorectal cancer patients (Lauscheke 2002).

In animals experiments, some authors found a decrease of colorectal cancer size and a decrease in lipid peroxidation (MDA) after the administration of luteolin (Ashokkumar 2008, Maniu 2005), and curcumina (Sharma 2001)

Gastric cancer

Human gastric cancer is a common disease and one of the leading causes of cancer mortality worldwide (Borrego 2012). In 1990, it was the fourteenth cause of death all over the world and despite a general decline in its incidence, projections indicate that the annual number of new cases will grow significantly in the developing world during the next few decades as a result of aging. Most gastric cancer is diagnosed at an advanced stage and survival is uniformly poor, usually no more than 15% at 5 years. (Bakan 2002)

Gastic cancer formation is a multifactorial process and there are many possible mechanisms leading to gastric carcinogenesis. Growing evidence indicates that reactive oxygen species (ROS) are associated with different steps of carcinogenesis, either through structural DNA damage, interaction with oncogenes or tumor suppressor genes or immunological mechanisms. For a variety of human cancers, chronic infection and inflammation have long been recognized as risk factors. It has been suggested that active oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical generated in inflamed tissues, can damage target cells, resulting in DNA damage and being able to contribute to tumor development. Growing evidence indicate that nitric oxide, an unpaired electron and its derivatives produced by activated phagocytes, may also play a role in the multistage carcinogenesis process. Nitric oxide is known, togheter with other ROS, to induce cytotoxicity and cytostasis. (Bakan 2002)

Gastric adenocarcinoma accounts for more than 95% of gastric tumors. Sporadic gastric tumors are known to be linked to a variety of etiological factors, such as diet, alcohol and tobacco habits, as well as *Helicobacter pylori*-induced inflammation, all of which may be related to underlying production of ROS and DNA damage. The generation and increase of ROS and secondary DNA oxidative damage are also suggested to be related to the damage and malignant transformation of gastric mucosa. Additionally, increased expression of human DNA repair genes has been reported in digestive tract tumors.(Borrego 2013)

Helicobacter pylori, a microaerophilic spiral bacterium, is the major cause of chronic gastritis and H. pylori associated gastritis is an established risk factor for gastric cancer. However, not everyone infected with H. pylori develops gastric cancer. One of the mechanisms by which H. pylori gastritis leads to gastric cancer is the generation of free radicals due to inflammatory response to DNA with subsequent DNA damage. Variety of DNA lesions and malignant transformations, which promote carcinogenesis by generation of genotoxic products like malondialdehyde (MDA), are produced. H. pylori infection increases free radical production. A delicate balance between generation of free radicals and endogenous as well as exogenous antioxidants like superoxide dismutase (SOD) and ascorbic acid is of critical importance for physiological functioning of the cell. When produced excessively or during deficient antioxidant defences, free radicals can begin lipid peroxidation and DNA damage, which leads to cellular destruction, chromosomal aberration, and finally cancer. (Khanzode 2003)

Our review of the literature confirm the association between gastric cancer and increase of MDA levels as shown in detail in next tables.

Author	Journal	Year	Country	Population	MDA	Increase of MDA levels (cancer pts versus controls)
Mosina LM	Klin Med (Mosk)	2011	Russia	-	-	x
Ilhan N.	World Gastroenterol	2004	Turkey	K: 43 Control group: 23	Plasmatic concentration	x
Khanzode S. D.	Cancer Letters	2003	India	K: 30 Control group: 40	Plasmatic concentration	x
Bakan E.	Jpn J clin Oncol	2002	Turkey	K: 38 Control group:24	Plasmatic concentration (TBARS)	x
Choi M.	Cancer Letters	1999	South Corea	K: 59 Control group: 44	Plasmatic concentration	x
Vainsshtein SG	Vopr Onkol	1984	Russia	K: 25 Control group: 14	-	x

Table 9. *MDA and gastric cancer (case-control studies conducted on humans, literature review)*

The table summarizes the case-controls studies selected according to our review strategy. Full text was not available for the articles of Mosina 2011 and Vainsshtein 1984. For this reason some information are lacking.

Author	Journal	Year	Country	Sample (KT/NT)	MDA	Increase of MDA in cancer sample
Borrego s.	Int J Mol Sci	2013	Spain	28/28	Tissues concentration; evaluation of leucocytes and urine adducts levels	x
Kekec Y.	European Journal of International Medicine	2009	Turkey	25/25	Tissues concentration	x
Batcioglu K.	Cancer Invest	2006	Turkey	-	Tissues concentration	x

Table 10. *MDA and gastric cancer (laboratory studies where sample of neoplastic tissues are compared to normal ones, literature review)*

KT*: sample of neoplastic tissue; NT§: sample of normal adjacent tissue

The table summarizes the laboratory studies where sample of neoplastic tissues are compared to normal ones,selected according to our review strategy. Full text was not available for the articles of Batcioglu 2006. For this reason some information are lacking.

Also other interesting experiments study the correlation between MDA and gastric cancer, even if from different points of view.

From the literature review we have performed, appears clear that the relation between MDA and gastric cancer is studied less than that for breast and colon cancers.

With regards to antioxidants in cancer tissues compared to normal ones, or in subjects with cancer compared to healthy ones, it is confirmed the same trend shown for the other two tumours. Discordant data are available in literature and some authors found also discordant levels of different antioxidants in the same sample or population. (Batcioglu 2006, Borrego 2013, Choi 1999, Dursun 2006, Khanzode 2003)

MDA levels get higher with the increase of cancer staging (Bakan 2002, Ilhan 2004), even if in some studies no significant differences were found (Dursun 2006)

Borrego found that after gastrectomy there is a normalization of MDA adducts in patients with gastric cancer.(Borrego 2012)

Ascorbic acid appears to act as a free radical scavengers and seems able to protect normal mucosa from evolution of gastric cancer (Drake 1996, Sun 2006)

The role of Helicocbater pylori in the evolution of gastric cancer trough chronic gastritis, is studied by some authors and the correlation with a progressive enhancement of MDA is detected (Farinati 1998, Ilhan 2004, Velazquez-Guadarrama 2007)

Breast cancer

Breast cancer is the main cause of cancer-related deaths among women worldwide, representing 14 % of cancer deaths in 2008. It is a complex and heterogeneous disease, and its prognosis and biological behavior are dependent by several tumor and host characteristics. Flogosis status plays a leading role in the immunomodulation of the tumor environment, as cytokines can have a stimulatory or suppressive effect, affecting prognosis and the response to therapy.

Recently researchers proposed a breast cancer molecular classification system based on several parameters (in detail tissue expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2)). On the basis of immunohistochemical staining and gene expression profiles, breast tumors are divided into 3 major subtypes:
-luminal tumors (subtype A and B) which are characterized by high expression levels of hormonal receptors (ER and PR) and represent roughly 70% of invasive breast cancers. The luminal A subtype has a better prognosis than the luminal B subtype owing to a lower level of expression of the proliferation marker Ki-67.
-the HER-2-positive subtype which is characterized by high expression levels of HER-2 and negative expression of ER and PR and comprises approximately 15 % of invasive breast cancers.
-triple-negative tumors (or basal-like) which show negative expression of HER-2, ER and PR and are described as the subtype with the worst prognosis.
Breast cancer therapy strategies are based on this classification as well as clinical factors, which are used to categorize patients in order to achieve the most adequate and effective treatment response. Breast cancer is an inflammatory disease and its progression involves changes in redox metabolism, which yield to cellular injury and irreversible damage to DNA caused by reactive species and cytokines. The involvement of estrogen, HER-2 and progesterone receptors in oxidative stress-mediated inflammatory pathways allow to hypothesize that the combination of such receptors (which identify specific molecular subtypes to human breast tumors) might lead to a distinct patterns of cytokine stimuli. Moreover it explains the singular behavior of each tumor subtype, allowing future specific therapeutic interventions. Knowledge of these processes is important because certain breast cancer subtypes present a very aggressive behavior and often result in increased mortality due to the lack of therapeutic options (Herrera 2012).

90% of mortality in breast cancer is associated with metastatic progression or relapse in patients. Critical stages in the development of aggressive breast cancer include the growth of primary tumors and their ability to spread to foreign organs and form metastases, as well as the establishment of an independent blood supply within the new tumors (Laino 2009).

Because the etiology of the main part of human breast cancers is unknown, preventive strategies for breast cancer have been difficult to identify There is growing evidence from animal and human systems implicating a role of oxidative stress and lipid peroxidation in the development of breast cancer. In chemical carcinogen-induced mammary tumor animal models, high-fat diets are associated with higher tumor incidence, and this trend is reduced by antioxidants. In human studies, elevated levels of cholesterol peroxides, known to be direct-acting mutagens, were observed in the breast fluid of breast cancer patients. Increased levels of MDA, an end product of lipid peroxidation, were detected in the urine of women with mammographic dysplasia (a condition associated with increased risk for breast cancer) and in the serum of breast cancer patients compared with non cancer controls. Tamoxifen,

an antiestrogen used in the treatment and chemoprevention of breast cancer, has been found to be an inhibitor of lipid peroxidation and has also been demonstrated to decrease serum levels of MDA in cancer patients. One consequence of oxidative stress and lipid peroxidation is the formation of DNA adducts. Since DNA is understood to be the target molecule for carcinogens, endogenous DNA adducts derived from oxidative stress, lipid peroxidation, and other sources have been proposed to contribute to the etiology of human cancers. With regard to breast cancer, a higher ratio of carcinogenic hydroxy adduct of DNA was detected in cancerous breast compared to normal breast tissue of noncancer., Furthermore, the levels of oxidative DNA damage in peripheral nucleated blood cells were significantly reduced by a low-fat diet intervention in women at high risk for breast cancer . All these observations strongly support the hypothesis that oxidative stress play a role in breast cancer etiology.(Wang 1996)

Our review of the literature confirm the association between breast cancer and the increase of MDA levels as shown in detail in next tables.

Author	Journal	Year	Country	Population	MDA	Increase of MDA levels (cancer pts versus controls)
Gupta RK	Asian Pac J Cancer Prev	2012	Nepal	K: 30 Control group: 100	-	x
Peluso M	Free Radic Res	2011	Italy	K: 22 Control group: 13	Adducts	x
Kedzierska M.	Gen Physiol Biophys	2010	Poland	-	-	x
Sinha	Indian Journal of Xancer	2009	India	K: 30 Control group: 20	Plasmatic concentration	x
Laino do Val de Carneiro	J Cancer Res Clin Oncol	2009	Brazil	K:59 Control group: 76	-	x
Chandramathi	J.Cancer Research and Clinical Oncology	2008	India	K: 101 Control group: 95	Urinary concentration	Reduction of MDA levels in K versus controls
Rajneesh C P	Singapore Med J	2008	India	K:40 Control group: 40	Plasmatic concentration	x
Shashar S.	Asian Pacific J Cancer Research	2008	Malaysia	K: 57 Control group: 139	Plasmatic concentration	x
Sener D. E.	Cell Biochem Funct	2007	Turkey	K: 56 Control group:18	Plasmatic concentration	x
Mannello F.	Int J. Cancer	2007	Italy / Columbia	K:24 Control group: 113	NAF concentration	Reduction of MDA levels in K versus controls
Gonenc A.	Cell Biology International	2006	Turkey	K: 15 Control group:15 (patients with mammary	Plasmatic concentration and in tissues	Reduction of MDA levels in K versus controls

				benign lesions)		
Yeh C-C	Clinica Chimica Acta	2005	Taiwan	K:117 Control group: 117	Plasmatic concentration	x
Faruk Tas	Medical Oncology	2005	Turkey	K: 40 Control group: 10 (patients with mammary benign lesions)	Plasmatic concentration	x
Khanzode SS	Free Radical Res	2004	India	*Full text not avaiable*	-	x
Akbulut H.	Cancer Detection and Prevention	2003	Turkey	K:10 Control group:10	Plasmatic concentration	x
Kumaraguruparan R.	Clinical Biochemestry	2002	India	K:30 Control group: 30	Plasmatic concentration (TBARS)	x
Gonenc A.	J of Clinical Pharmacology and Therapeutics	2001	Turkey	K: 26 Control group:41	Plasmatic concentration	x
Ray G	Br J Biomed Sci	2001	India	K;45 Control group: 45	Plasmatic concentration	Significantly higher in first stages of cancer; in the most serious stages decresce
Ray G	Breast Cancer Res Treat	2000	India	K: 54 Control group: 54	Plasmatic concentration	x (stages II, III, postmenopause, premenopause)
Huang Y-L	Clinical Biochemestry	1999	Taiwan	K: 35 Control group: 35	-	x
Alagol H	Aust N Z J Surg	1999	Turkey	*Full text not available*	-	Reduction of MDA levels in K versus controls
Seven A.	Cancer Biochem Biophys	1998	Turkey	K: 23 Control group: 15 (patients with mammary benign lesions)	Plasmatic concentration	Reduction of MDA levels in K versus controls
Wang M	Cancer Epidemiol Biomarkers Prev	1996	USA	K:51 Control group :28	Adducts	x
Hietanen E.	Eur J Clin Nutr	1994	Finland	-	Plasmatic concentration	-
Gerber M	Cancer	1989	France	K :120 Control group: 109	Plasmatic concentration	Reduction of MDA levels in K versus controls

Table 11. *MDA and breast cancer (case-control studies conducted on humans, literature review)*

The table summarizes the case-controls studies selected according to our review strategy. Full text was not available for the articles of Gupta 2012, Kedzierska 2010, Huang 1999, Alagol 1999, Hietanen 1994. For this reason some information are lacking.

Also other interesting experiments study the correlation between MDA and breast cancer, even if from different points of view. We report the most significant ones.

Akbulut and colleagues investigate the diurnal variations of MDA in patients with early breast cancer (BC). The daily average MDA levels were higher than that of controls and the plasma MDA levels of the patients showed significant diurnal variations (highest levels at 20:00, lowest at 4:00). The results suggest that phase differences in daily variations of lipid peroxidation may play a role in carcinogenesis (Akbulut 2003).

MDA levels were detected higher in women with extensive mammographic dysplasia than healthy controls. This results suggest that breast dysplasia may be associated with lipid peroxidation; mutagenic products generated by this process may influence breast cancer risk. (Boyd 1990)

With regards to antioxidants in breast cancer tissues compared to normal ones, or in subjects with breast cancer compared to healthy ones, contrasting data are available in the literature reviewed as seen for CRC. Some authors found also discordant levels of different antioxidants in the same sample or population. (Gupta 2012, Hietanen 1994, Huang 1999,Kumaraguruparan 2002, Rajneesh 2008, Ray 2000, Sener 2007, Seven 1998, Sinha 2009, Faruk Tas 2005, Shahar 2008, Gonenc 2006).

MDA levels result increasing with the worsening of the TMN stage (Khanzode 2004, Sinha 2009, Ray 2000)

Herrera and collegues found that inflammatory status varies in distinct ways due to molecular subtype of breast cancer (Herrera 2012).

In a study conducted on mice with breast cancer, mice were exposed to acute hypoxia for 6 weeks. Tumor hypoxia is known to be a poor indicator, predictive of increased risk of metastatic disease and survival. Even if in this model the exposure to acute hypoxia, did not translate into significant changes in cancer progression at the primary or metastatic levels, it causes an increase in lipid peroxidation products levels (Kalliomaki 2008)

The antiestrogenic therapy with tamoxifene alone or associated with riboflavin/niacin/CoQ10 leads to a reduction of lipid peroxidation (lower MDA levels).(Perumal 2005)

Martin and collegues tested on Salmonella thyphimurium and Escherichia coli the genotoxicity of breast lipid tissue, but did not find any conclusive correlation (Martin 1996).

Some authors try to explain the absence of MDA increase in the presence of breast cancer that some studies detetected. (Mannello 2007, Gago-Dominiquez 2005, Seven 1998, Gonenc 2006) The role of lipid peroxidation in BC initiation is controversial; it is considered harmful and carcinogenic, but it also regulates growth inhibition and cell death. As it is clearly shown in the table, several studies have found enhanced lipid peroxidation and depletion of antioxidants in the plasma of BC patients, suggesting that increased oxidative stress may be related to human breast carcinogenesis. Moreover, an increased content of lipid peroxidation products was found in BC tissue, supporting the hypothesis of breast tumorigenesis by in situ oxidative stress. On the other hand, some reports suggest that lipid peroxidation may represent a protective mechanism in BC through the induction of apoptosis, thereby maintaining the balance between breast epithelial cell growth and death,

supporting those data according to which lipid peroxidation in normal tissue or in tissue of benign breast disease is greater than in BC (Mannello2007).

It is interesting that patients with breast cancer, presenting the genetical mutation BRCA1, did not have increased levels of indicators of oxidative stress as expected. BRCA1 involved in cellular antioxidant response by inducing the expression of genes of the antioxidant defence system and thus conferring resistance to oxidative stress. (Kotsopoulos 2008)

Aim of the study

Most studies in the field of chemical carcinogenesis and molecular epidemiology have focused on the analysis of DNA adducts induced from exposure to environmental carcinogens, particularly those contained in cigarette smoke and industrial and urban air pollution.

Experimental and epidemiological data suggest that there are lots of endogenous metabolic products that have carcinogenetic properties as well, but they are not yet extensively studied. There is a growing evidence that endogenously generated compounds may play an important role in the carcinogenic process, especially through the formation of DNA adducts (Marnett 1999). In fact, an important development of the past cancer research has been the discovery that a significant amount of DNA damage, up to about one DNA adduct for 10^6 bases (Chaudhary 1994), is arising from natural and endogenous sources, at levels comparable to the highest levels of DNA adducts found in tissues from individuals exposed to environmental carcinogens. Moreover, the endogenous formation of DNA adducts could be involved in the etiology of certain human cancers, including breast, gastric and colon cancers (Everett 2001).

Moving from these basis, we choosed to examine in depth malondialdehyde, a lipid peroxidation product and one of such endogenous agent, which seems to be a promising biomarker for free radical damage. (Lasheras 2003).

The literature reviewed confirmed that this field has not been extensively studied yet and that there is a lot to investigate about these topics.

Firstly, we conducted a study in a representative sample of the general population of the city of Florence. We evaluated the determinant (environmental, lifestyle, dietetic ones) of MDA adduct level in these clinically healthy subjects.

Secondly, we conducted a case-control study, comparing the MDA adduct levels detected in the general population sample (controls) and those detected in subjects affected by colon, breast, or gastric cancer (cases).

Materials and Methods

Subjects

A sample of 500 subjects (250 males and 250 females) aged 40-64 years was randomly selected on 1 January 1997 from the computerized population registry of residents in the city of Florence. A standardized contact protocol was developed: each selected subject received a letter of invitation with a brief description of the aims of the study and, subsequently, was contacted by phone by an interviewer who provided more information on the study protocol and, if the subject agreed to participate, made an appointment for a visit. In order to increase the participation in the study, a panel of common blood tests was offered free of charge. The enrolment took place mostly during 1997.

Among the 500 sampled subjects, 362 (72.4%) accepted to participate; the response rate was similar in men (178/250; 71.2%) and women (184/250; 73.6%). Among the 138 nonresponders, 108 (21.6%) refused to participate, 24 (4.8%) were seriously ill, and 6 (1.2%) could not be traced. The mean age of participants was 52.9 years (53.6 years in males and 52.3 in females).

All participants, after signing an informed consent form, were asked to agree to the same protocol followed by the EPIC volunteers. Each participant also provided a fasting blood sample. (Masala 2003).

For each volunteer the following information and measurements were collected usually on the same day of enrolment:

1) detailed demographic information for the follow-up of each subject;

2) a short questionnaire on recent use of pharmaceutical drugs, current smoking habits and occupation;

3) anthropometric measurements (weight, height, sitting height, waist and hip circumferences) and blood pressure according to a standardized protocol with periodic quality control procedures (including between- and within- observer variability);

4) a self-administered dietary questionnaire, with pictures of different portion sizes of a series of selected foods;

5) a self-administered life-style questionnaire (smoking, alcohol consumption, reproductive and medical history, environmental tobacco smoke, physical activity).

The food frequency questionnaire had been specifically developed for the Italian dietary pattern and tested in a pilot phase. Dietary information on the frequency of consumption of more than 120 foods and beverages obtained by the questionnaire was checked, coded, computerized by optical reading and then transformed into estimates of intake of 40 nutrients according to specifically developed Italian Food Tables (Palli 2003). The modified Mediterranean diet score by Trichopoulou and colleagues was calculated. Values of zero or one were assigned to each of nine specific dietary items, using as cut-off values the sex specific medians among the participants. People whose consumption of presumed beneficial components (vegetables, legumes, fruits, cereals, fish) was below the median consumption, were assigned a value of zero, and a value of one otherwise. People whose consumption of presumed detrimental components (meat and dairy products) was below the median consumption were assigned a value of one, and a value of zero otherwise. A value of one was given to men consuming from 10 g to less than 50 g of ethanol per day and to women consuming from 5g to 25g. For lipid intake, the ratio of the sum of monounsaturates and polyunsaturates to saturates was calculated. This modified Mediterranean diet score can take a value from zero (minimal adherence) to nine (maximal adherence). (Trichopoulou 2005)

A standardized life-style questionnaire (specifically printed in two versions for men and women) was also completed by each participant. The questionnaire represents the Italian translation of a common English version developed at the European level for EPIC project. Detailed information was collected on reproductive history, physical activity, smoking history, alcohol consumption, medical history, occupation, educational level and other socio-economic variables. The section on smoking history collected specific information for separate time periods (at the age of 20, 30, 40 and 50 years, approximately), including the cumulative length of periods of smoke cessation for both current and former smokers. A detailed physical activity section was included in the life-style questionnaire, with specific questions on physical activity related to the current job (sedentary, mainly standing, manual work, very heavy manual work), floor climbing, walking, biking, gardening, and physical fitness. Information on these latter activities was collected relative to winter and summer months. Women were also asked a specific question regarding daily hours of house cleaning activities.

A total physical activity index was developed according to the recommendation of the EPIC Physical Activity research group (Lahmann 2007).

Metabolic equivalents (METs) for each specific activity were derived from the Compendium of Physical Activities (Ainsworth 2000) and assigned as follows: 3.0 for walking, 6.0 for biking, 4.0 for gardening, 6.0 for "fitness" activities, 4.5 for home repair (do-it-yourself work), 3.0 for housework and 8.0 for stair climbing. These mean MET values were obtained by estimating the average of all comparable activities in the Compendium. For each recreational and household activity, the hours/week reported were multiplied by the specific MET value, and specific MET-hours/week were obtained to provide an estimated measure of energy expenditure.

Through a cross-classification of quartiles based on the distribution in the population of total MET hours/week in leisure time, with the occupational PA categories a score of total activity level (PA total index) was created with the following categories : "inactive", "moderately inactive" "moderately active" and "active".

After coding and quality control procedures, all available questionnaires were computerized by optical reading. A computerized data base with the dietary and lifestyle information was completed for all participants and is currently available for analysis. (Palli 2003)
Each participant provided a fasting blood sample. (Masala 2003).

Following a common protocol (the same of the European project) and using identical equipment, 30 mL of blood were collected for over 362 participants, mostly while fasting. The blood samples were processed and centrifuged in a dedicated laboratory, in the same day of collection, and were divided into 28 aliquots of 0.5 mL each (12 plasma, 8 serum, 4 concentrated red blood cells, and 4 buffy coat) using an automatic aliquoting and sealing machine specifically developed by BICEF (Cryo-Bio Straw). The aliquots, after a slow freezing process at -80 °C, were divided into two series and stored separately in liquid nitrogen tanks at -196 °C. The samples are currently stored in a local biological bank specifically developed in Florence. A specific computer program was developed to automatically assign the position in the liquid nitrogen container to each subject's aliquots. This program allows the management of the bank for the retrieval of specimens in the ongoing research projects. The collection and storage at low temperature of blood and other biological samples constitutes a major characteristic of the new generation of prospective studies on cancer and diet-related hormonal and nutritional factors. (Palli 2003)

For the MDA adduct analyses, we retrieved the buffy-coats from the cryogenic tanks. The estimates on MDA adducts were available only for 313 subjects because the buffy-coats were not available for some subjects and due to some technical problems.

213 subjects residing in the city of Florence, with a new diagnosis of cancer (75 breast cancer, 87 stomach cancer and 51 colon cancer) were also enrolled in the frame of an ongoing case-control study carried out in the metropolitan area of Florence. These cases were recruited in the period 2000-2009 when admitted to the Surgery Departments of the main hospitals in the area (particularly, Careggi). All cases had a confirmed histological diagnosis They had an interview with a nutritionist, who explained in detail the purpose of the study and what the participants were requested to do. All participants signed an informed consent form and provided a blood sample. Information and blood sample were collected before surgery and the start of any therapy. The cases followed the same protocol previously followed by the EPIC volunteers and for the Florence population sample as regard the compilation of the two EPIC questionnaires. The anthropometric measurements were self reported by patients

Out of 213 cases who joined the study, only 204 (71 breast cancer, 82 stomach cancer, 51 colon cancer) completed the protocol in all its parts. The blood sample and the buffy-coats were available for all 213 subjects.

Laboratory measurement adducts

A reference adduct standard was prepared: calf-thymus (CT)-DNA was treated with 10 mM MDA (ICN Biomedicals, Irvine, CA, USA). MDA treated CT-DNA was diluted with untreated DNA to obtain decreasing levels of the reference adduct standard to generate a calibration curve.

DNA was extracted and purified from buffy-coats using a method that requires digestion with ribonuclease A, ribonuclease T1 and proteinase K treatment and extraction with saturated phenol, phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1) and ethanol precipitation. DNA concentration and purity were determined using a spectrophotometer. DNA samples were subsequently stored at -80 °C.

DNA adducts in MDA treated CT-DNA sample were analyzed by mass spectrometry (Voyager DE STR from Applied Biosystems, Framingham, MA), through the following sequence of steps:
(1) reaction of DNA with $NaBH_4$ followed by precipitation with isopropanol;
(2) digestion with snake venom phosphodiesterase and nuclease P1;
(3) extraction of DNA adducts that are less polar than normal nucleotides on an OASIS cartridge (Waters Corp.);
(4) tagging with an isotopologue pair of benzoylhistamines (d_o and d_4) in a phosphate-specific labeling reaction in the presence of carbodiimide;
(5) removal of residual reagents by ion exchange solid-phase extraction;
(6) resolution of tagged adducts by capillary reversed-phase HPLC with a collection of drops onto a MALDI plate;
(7) addition of matrix (α-cyano-4-hydroxycinnamic acid);
(8) analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

The peripheral blood levels of exocyclic DNA adducts, indicated from M_1dG, a biomarker of oxidative stress and LPO, were measured using a modified version of the ^{32}P-DNA postlabeling assay. In detail, DNA (2 μg) was hydrolyzed by incubation with micrococcal nuclease (21.45 mU/μl) and spleen phosphodiesterase (6.0 mU/μl) at 37°C for 4.5 h. Hydrolyzed DNA was treated with nuclease P1 (0.1 U/μl) at 37°C for 30 min. After enzymatic treatment, samples were incubated with 25 μCi of carrier-free [γ-^{32}P] ATP (3000 Ci/mM) and polynucleotide kinase T4 (0.75 U/μl) to generate ^{32}P-labeled adducts at 37°C for 30 min.

^{32}P-labeled adducts were applied on polyethyleneimine cellulose thin-layer chromatography plates (Macherey-Nagel, Germany) and processed as previously described. This chromatographic modification of the ^{32}P-postlabeling method has been developed from our laboratory for the specific detection of this specific kind of exocyclic DNA adducts by using a low-urea solvent system known to be effective for the detection of low molecular weight and highly polar DNA adducts. In brief, ^{32}P-labeled products were applied to the origin of chromatograms and developed with 0.35 MgCl2 up to 2.0 cm filter paper wick. Plates were developed in the opposite direction with 2.1 M lithium formate, 3.75 M urea, pH 3.75, and then run at the right angle to the previous development with 0.24 M sodium phosphate, 2.4 M urea, pH 6.4.

Subsequently, the detection and quantification of M_1dG adducts and total nucleotides (nt), i.e. diluted samples that were not treated with NP1, were performed by storage phosphor imaging techniques employing intensifying screens from Molecular Dynamics (Sunnyvale,

CA, USA). The intensifying screens were scanned using a Typhoon 9210 (Amersham). Software used to process the data was ImageQuant (version 5.0) from Molecular Dynamics.

After appropriate background subtraction, the levels of M_1dG adducts were expressed such as relative adduct labelling (RAL) = pixels in adducted nucleotides / pixels in nt. The levels of M_1dG adducts were corrected across experiments based on the recovery of reference standard.

The presence of the M_1dG adduct in this sample was confirmed by MALDI-TOF-MS. A calibration curve was then set up by diluting this sample with untreated CT-DNA and measuring the decreasing level of M_1dG (r-squared = 0.99).(Peluso 2011, Munnia 2007)
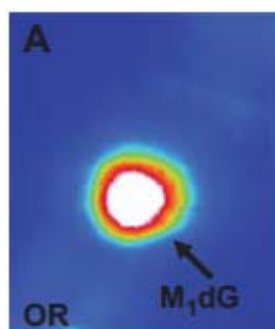

Figure 8. *Typical autoradiograms of malondialdehyde deoxyguanosine adduct in 10 mM malondialdehyde-treated calf thymus DNA (From Peluso 2011)*

Statistical analyses

All questionnaires filled in by the general population sample and by cancer patients were checked and coded by trained dieticians and computerized. The data collected from the self-administered Food Frequency Questionnaire were transformed into estimates of intake of a series of nutrients according to specifically developed Italian food tables.

Simple descriptive analyses of selected social and demographic characteristics and life-style habits were performed separately in men and women identified in the representative sample of residents in Florence.

Differences in selected individual characteristics, anthropometric measurements between men and women in the Food Frequency Questionnaire (FCS) were evaluated by means of the chi-squared test for categorical variables and Wilcoxon's rank sum test for continuous variables.

A descriptive analysis was initially performed to explore the relationship between individual characteristics and M_1dG adducts. All statistical analyses were performed on log-transformed data to stabilize the variance and normalize the distribution of M_1dG adducts.
To this end, we used the geometric means (GM) of M1dG adducts obtained by a covariance analysis including terms for sex and each individual characteristic. P-value were obtained by Dunnet test. As regard the association with dietary variables we evaluated a specific dietary score (Modified Mediterranean Diet Score, MMDS), calculated as suggested by Trichopoulou (BMJ 2005) according to an estimate based on the sex-specific median values of consumption (calculated on the entire EPIC Florence based on 13957 subjects) of eight food items (vegetables, legumes, fruit, cereals, fish, ratio MUFA + PUFA/SFA, red meat, dairy products), and sex-specific cut-off alcohol intake values. The score can range between 0 (minimal adherence to the MMDS) to 9 (maximum adherence to the MMDS).We used in our statistical analyses different forms of this score (continuum, below/above the median value, and specific categories corresponding to three levels of diet quality: 0-3 low, 4-6 medium and 7-9 high quality).

Then a univariate and multivariate regression analysis were performed in the healthy population sample.
In the univariate setting, the mean levels of DNA adducts across the levels of each individual characteristic, were compared by analysis of variance, in order to evaluate the association between each potential determinant and MDA adducts levels.
The multivariate analysis was then performed adjusting for the concomitant effect of all variables included in the model. A multivariate regression model including terms for sex (F *vs* M), age (continuum), BMI (overweight, obesity *vs* normal weight), smoking status (smoker *vs* non smoker), physical activity level (at 4 levels: inactive, moderately inactive, moderately active, active), education level (degree *vs* other), total caloric intake (continuum), and *MMDS* (in different forms, by separate models) was carried out to estimate the effect of each parameter on the outcome adjusting for the concomitant effect of the other parameters included in the model. All regression models were carried out in the whole series and separately by sex.
The regression analyses performed in females were also adjusted by menopausal status and age at menarche.

Simple descriptive analyses of selected social and demographic characteristics and life-style habits were performed for the cancer population affected sample, subdivided in breast, colon,

gastric cancer affected population samples, in comparison to the general healthy population sample.

Then breast, colon, gastric cancer cases , were compared to the general healthy population sample (control group) with regards to MDA adducts levels

The statistical analyses were performed on log-transformed data and the Mann Whitney U test was used to compare the mean MDA adducts levels. For colon and gastric cancer studies the adjustment variables were sex (male/female), age (years), BMI (normal/overweight/obese), Education level (degree/other), Mediterranean Modified Diet Score (Low/Medium/High), Physical Activity Index (inactive, moderate inactive, moderate active, active), Smoking habit (Yes/No).

For breast cancer study only crude and age adjusted mean MDA adduct level were calculated.

All the analyses were performed using SAS (SAS institute, Cary, North Carolina) statistical program. A p-value <0.05 (two tailed) was considered statistically significant for all tests.

Results

General population

Firstly we are going to expose the results regarding the description of the general population sample and the emerging correlations between M_1dG and potential determinants.

Selected individual characteristics of the 313 healthy subjects of the city Florence sample are shown in Table 12.

Table 12. *Description of the study subjects by sex.*

	Men	**Women**	
Total	**139**	**174**	**p-value***
	Mean (SD)	**Mean (SD)**	
Age (years)	53.5 (6.7)	52.4 (7.4)	0,16
Height (cm)	172.19 (6.73)	158.32 (6.26)	<0,0001
Weight (kg)	79.19 (12.72)	63.41 (12.76)	<0,0001
BMI (kg/mt^2)	26.68 (3.85)	25.31 (4.87)	<0,0001
Waist (cm)	92.49 (10.57)	78.24 (13.88)	<0,0001
Hip (cm)	98.56 (7.33)	98.68 (9.48)	0,46
WHR	0.94 (0.07)	0.79 (0.11)	<0,0001
	N (%)	**N (%)**	
BMI classes			0,0003
Normal	48 (34.5)	99 (56.9)	
Overweight	67 (48.2)	53 (30.5)	
Obese	24 (17.3)	22 (12.6)	
Physical activity°			0,31
Inactive	35 (25.2)	55 (31.8)	
Moderat. Inactive	46 (33.1)	61 (35.3)	
Moderat. Active	42 (30.2)	45 (26.0)	
Active	16 (11.5)	12 (6.9)	
Smoking status			0,16
smoker	59 (42.4)	60 (34.4)	
non smoker	80 (57.6)	113 (65.6)	
Education level			0,93
Degree	26 (11.5)	33 (19.0)	
Other	113 (88.5)	141 (81.0)	

° total physical activity index

*p-value from chi-square test or Wilcoxon test as appropriate

The sample was composed of 139 men and 174 women.

The mean age was similar between males and females (53,5; 52,4 respectively)

The mean height and mean weight in this representative sample were 172.19 cm and 79.19 kg in males and 158.32 cm and 63.41 kg in females, respectively. Men and to a lesser extent women tended to be overweight, the mean body mass index (BMI) being 26.68 and 25.31, respectively.

According to BMI classes, most men were classified as overweight (48,2%) and women as normal (56,9%); 17.3% of males and 12.6% of females were obese. The proportion of current smokers was 42,2% in males and 34.4% in females.

The physical activity level considering working, home and leisure time was higher in men than in women(men, defined as moderately active and active, represent the 41,7%, whereas women only the 32,9%).

About the educational level, only the 11,5% of the men and the 19% of the women had a degree.

The figure below represents the distribution of M_1dG adducts per 10^8 normal nucleotides in the series of 313 healthy subjects of the Florence City Sample. The mean value of 6 M_1dG adducts per 10^8 normal nucleotides was 2.18 (SD 1.58), with a range between 0.4 and 12.0 The majority of the values are included between 1 and 6 M_1dG (more than 70.0%).

Figure 9. *Distribution of M_1dG adducts per 10^8 normal nucleotides in the series of 313 healthy subjects of the Florence City Sample.*
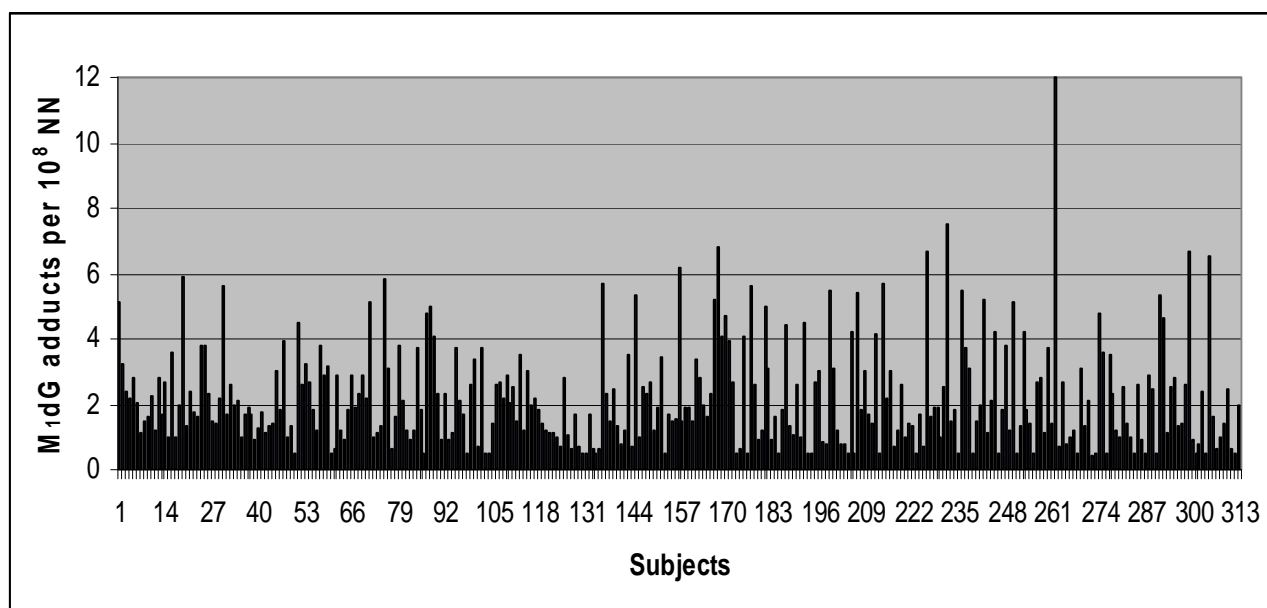
The crude means values of M_1dG per 10^8 normal nucleotides according to selected individual characteristics highlight a significant difference only for sex (2.14 ± 1,88 versus female 2.21± 1.29, p 0.004). Moreover the present analysis suggest that there could be an inverse correlation between M_1dG and the Modified Mediterranean Diet Score (MMDS). In fact, the three MMDS categories (low, medium, high) show a decrease of the means of M_1dG at the increase of the categories (from low to high). The p-value is not significant (p=0.22), but this trend suggest further investigation in this direction.

Table 13. *Crude means values of M_1dG per 10^8 normal nucleotides according to selected individual characteristics*

Characteristics	N	Mean ± SD			p-value°
Sex					
Male	139	2,14	±	1,88	**0.004**
Female	174	2,21	±	1,29	
Age (yrs)					
<50	123	2,25	±	1,60	
51-60	131	2,10	±	1,62	
>60	59	2,23	±	1,46	
					0.83
BMI class					
Normal	147	2,22	±	1,39	
Overweight	120	2,10	±	1,53	0.75
Obese	46	2,25	±	2,19	
Weight (quartiles*)					
I	78	2,31	±	1,54	
II	79	2,16	±	1,39	0.78
III	78	2,12	±	1,73	
IV	78	2,15	±	1,67	
Height (quartiles*)					
I	77	2,27	±	1,86	
II	77	2,23	±	1,47	0.85
III	80	2,10	±	1,43	
IV	79	2,14	±	1,55	
Waist (quartiles*)					
I	77	2,33	±	1,53	
II	75	2,00	±	1,28	0.37
III	82	2,45	±	1,94	
IV	77	1,97	±	1,44	
Hip (quartiles*)					
I	77	2,43	±	1,59	
II	81	2,06	±	1,31	0.43
III	76	2,01	±	1,45	
IV	77	2,28	±	1,90	
WHR (quartiles*)					
I	79	2,09	±	1,36	
II	78	2,26	±	1,80	0.93
III	79	2,24	±	1,50	
IV	75	2,18	±	1,67	

Smoking status					
smoker	119	2,11	±	1,64	
non smoker	194	2,22	±	1,54	0.22
Education level					
Degree	59	1,92	±	1,27	
Other	154	2,24	±	1,63	0.27
PA ^					
Inactive	90	2,32	±	1,52	
Moderately inactive	107	2,18	±	1,49	
Moderately active	87	2,03	±	1,43	0.59
Active	28	2,23	±	2,39	
MMDS					
Low (0-3)	98	2,30	±	1,52	
Medium (4-6)	181	2,16	±	1,63	0..22 $
High (7-8)	33	1,96	±	1,47	

° from separate covariance analyses including terms for sex and each parameter listed in the table (Dunnet test used for covariance analysis)

* quartiles sex-specific;

^ total physical activity index

$ from a separate covariance analysis including terms for sex, kcal and *MMDS*

The mean values of estimated daily consumption of selected foods in the representative sample of residents in the city of Florence are shown in Table 14, according to the three category of the MMDS. The values for each item are expressed in g/day. Daily consumption of vegetables (all types combined), fruits, legumes, cereals and fish increase with the increasing of MMDS, and it is higher in the group "high MMDS" than in the others . An opposite trend results for the assumption of dairy products, red meat and alcohol: daily consumption of these items decrease with the increasing of MMDS, and it is lower in the group" high MMDS" than in the others.

Table 14. Mean values (± DS) of consumption of selected food groups by MMDS* category

Item (g/day)	MMDS		
	Low (0-3) **n:98**	**Medium (4-6)** **n:181**	**High (7-8)** **n:33**
Vegetables	133.8 (89.4)	205.1 (99.4)	254.5 (65.0)
Legumes	12.3 (8.5)	20.7 (16.3)	29.9 (13.7)
Fruit	243.0 (200)	318.6 (198.1)	370.6 (154.7)
Cereals	218.6 (95.9)	268.3 (133.5)	289.4 (107.3)
Fish	17.0 (12.7)	31.1 (19.1)	37.2 (15.4)
Dairy products	252.6 (215.6)	226.7 (238.6)	161.7 (102.5)
Red meat	86.8 (53.4)	76.4 (49.9)	73.2 (52.8)
Alcohol	18.9 (30.4)	17.8 (19.7)	15.7 (12.2)
Ratio MUFA+PUFA/SFA	1.6 (0.3)	1.8 (0.4)	2.0 (0.3)

* estimated according to a calculation, within the EPIC-Florence cohort, based on the sex-specific median values of consumption of vegetables, legumes, fruit, cereals, fish, ratio MUFA+PUFA/SFA, red meat, dairy products, and sex-specific cut-off alcohol intake values (from Trichopoulou, BMJ 2005)

The association between M_1dG per 10^8 normal nucleotides (log) and selected individual characteristics by a multivariate regression model (including terms for sex, age, BMI, smoke, physical activity, education level, total caloric intake and the *MMDS*) are shown in Table 15. A statistically significant positive association between sex (female) and M1dG was found (p-value 0.015). Borderline associations emerged with education level and physical activity (PA). An high educational level (degree versus other) was associated with lower levels of M1dG (p-value 0.053). A sedentary lifestyle was associated with higher levels of MDA adducts (p-value 0.051). The multivariate regression model suggests that there is an inverse association, although not statistically significant, between MMDS score and M_1dG levels (p-value 0.069).

Table 15. *Association between M_1dG per 10^8 normal nucleotides (log) and selected individual characteristics by a multivariate regression model, including terms for sex, age, BMI, smoke, physical activity, education level, total caloric intake and the MMDS: coefficient and p-values.*

Variable	Coefficient	p-value
Sex (female)	0.096	**0.015**
Age (continuum)	-0.0001	0.88
BMI Overweight	-0.038	0.35
Obesity	-0.059	0.28
Smoke Smoker *vs* no smoker	-0.061	0.10
PA (groups)	-0.039	0.051
Education level Degree *vs* other	-0.093	0.053
Kcal (continuum)	0.0001	0.62
MMDS Categories (0-3, 4-6, 7-8)	-0.053	0.069

To better explore the association between M_1dG and MMDS, we used different categorization of the MMDS finding a significant inverse association between M_1dG and the extreme MMDS category (MMDS=8) (p=0.046)

In the following section there is a description of the cases sample population. This description is conducted separately for each cancer population studied (colon, gastric and breast cancer).

Colon Cancer
Selected individual characteristics of the 51 colon cancer patients are shown in Table 16 and compared to the controls.
The sample was composed of 29 men and 22 women.
The population tended to be overweight; according to BMI classes, the 35,29% of the population sample was overweight and the 15,69% was obese. The proportion of current smokers was 82,3% and 34.4% of no smokers.
The physical activity level considering working, home and leisure time was moderately active for the 31,7% of the population sample and active only for the 9,80%. About the educational level, only the 11,76% had a degree.

Table 16. *Distribution of selected individual characteristics in colon cancer cases and controls*

COLON

Characteristics	Description	Cases N°	%	Controls N°	%	P-value*
Sex	M	29	56,86%	139	44,41%	0,0981
	F	22	43,14%	174	55,59%	
	Total	**51**	**100,00%**	**313**	**100,00%**	
BMI	Normal	25	49,02%	147	46,96%	0,9161
	Overweight	18	35,29%	120	38,34%	
	Obese	8	15,69%	46	14,70%	
	Total	**51**	**100,00%**	**313**	**100,00%**	
Education level	Degree	6	11,76%	59	18,85%	0,4478
	Other	45	88,24%	254	81,15%	
	Total	**51**	**100,00%**	**313**	**100,00%**	
PA ^	Inactive	11	21,57%	90	28,85%	0,7611
	Moderately inactive	19	37,25%	107	34,29%	
	Moderately active	16	31,37%	87	27,88%	
	Active	5	9,80%	28	8,97%	
	Total	**51**	**100,00%**	**312**	**100,00%**	
MMDS	Low (0-3)	14	27,45%	98	31,41%	0,0120
	Medium (4-6)	24	47,06%	181	58,01%	
	High (7-8)	13	25,49%	33	10,58%	
	Total	**51**	**100,00%**	**312**	**100,00%**	
Smoking status	Smoker	42	82,35%	194	61,98%	0,0047
	No smoker	9	17,65%	119	38,02%	
	Total	**102**	**100,00%**	**313**	**100,00%**	
Age (mean)		**52,87**		**61,51**		**<0,0001**

*p-value from chi-square test or Wilcoxon test as appropriate

Table 17. *Crude mean of M_1dG in colon cancer population versus controls*

	Mean M_1dG	N	Standard Dev.	P-value*
Controls	2,1812	313	0,10	< 0,0001
Colon cancer	3,5529	51	0,25	

*p value from Mann Whitney U test

Table 18. *Sex – and age- adjusted mean of M_1dG in colon cancer population and controls*

	Adj. Mean M_1dG	N	Standard Dev.	P-value*
Controls	2,1836	313	0,10	< 0,0001
Colon cancer	3,5373	51	0,27	

*p value from Dunnet test

Table 19. *Multiple adjusted* *mean of M_1dG in colon cancer population versus controls*

	Adj. Mean M_1dG	N	Standard Dev.	P-value*
Controls	2,2014	313	0,11	< 0,0001
Colon cancer	3,4439	51	0,28	

- ****adjusted for sex, age, BMI, smoke, physical activity, education level, total caloric intake and the *MMDS*

*p value from Dunnet test

The comparison of the crude mean of M_1dG in colon cancer population sample versus healthy controls sample show significantly higher levels of M1dG adducts in cancer patients than in controls (p< 0,0001). This difference is confirmed also when the M_1dG mean was adjusted for sex and age, and when the mean of M_1dG is adjusted for multiple factors (sex, age, BMI, smoke, physical activity, education level, total caloric intake and the *MMDS)*.

Table 20. *Crude Mean of M_1dG in colon cancer cases and control by categories of MMDS*

		Controls	Crude Mean		Cases	Crude Mean	
		N°	Mean M1dG	Standard Dev.	N°	Mean M1dG	Standard Dev.
MMDS	Low (0-3)	98	2,2959	0,1597	14	2,7785	0,7881
	Medium (4-6)	181	2,1643	0,1175	24	4,1458	0,6019
	High (7-8)	33	1,9560	0,2752	13	3,2923	0,8179
	Total	**312**			**51**		

Table 21. *Adjusted* Mean of M_1dG in colon cancer cases and control by categories of MMDS.*

		Controls	*Adj. Mean		Cases	*Adj. Mean	
		N°	Mean M1dG	Standard Dev.	N°	Mean M1dG	Standard Dev.
MMDS	Low (0-3)	98	2,3122	0,1611	14	2,8363	0,8422
	Medium (4-6)	181	2,1753	0,1180	24	4,2384	0,6434
	High (7-8)	33	1,8475	0,2798	13	3,0592	0,8998
	Total	**312**			**51**		

- *adjusted for sex, age, BMI, smoke, physical activity, education level

Then we conducted a comparison between the crude means of M_1dG of the colon cancer cases and those of the controls, in three categories of Modified Mediterranean Diet Score. The suggested inverse correlation between M_1dG and the Modified Mediterranean Diet Score (MMDS) found in the healthy control sample did not emerge in the cancer colon population sample. However, in each category of MMDS the M_1dG values were higher among cases compared to controls. The same comparison was conducted also considering the adjusted means of M_1dG of the colon cancer cases and those of the controls, with similar results.

Gastric Cancer

Selected individual characteristics of the 51 gastric cancer patients are shown in Table 22 and compared to the controls.

The sample was composed of 41 men and 46 women.

The population tended to be overweight; according to BMI classes, the 40,68% of the population sample was overweight and the 10,17% was obese. The proportion of current smokers was 85,06% and 14,94% of no smokers.

The physical activity level considering working, home and leisure time was moderately active for the 34,12% of the population sample and active only for the 7,06%. About the educational level, only the 2,30% had a degree

Table 22. *Distribution of selected individual characteristics in gastric cancer cases and controls*

STOMACH

Characteristics	Description	Cases N°	Cases %	Controls N°	Controls %	P-value*
Sex	M	41	47,13%	139	44,41%	0,6522
	F	46	52,87%	174	55,59%	
	Total	**87**	**100,00%**	**313**	**100,00%**	
BMI	Normal	29	49,15%	147	46,96%	0,6544
	Overweight	24	40,68%	120	38,34%	
	Obese	6	10,17%	46	14,70%	
	Total	**59**	**100,00%**	**313**	**100,00%**	
Education level	Degree	2	2,30%	59	18,85%	< 0,0001
	Other	85	97,70%	254	81,15%	
	Total	**87**	**100,00%**	**313**	**100,00%**	
PA ^	Inactive	16	18,82%	90	28,85%	0,2309
	Moderately inactive	34	40,00%	107	34,29%	
	Moderately active	29	34,12%	87	27,88%	
	Active	6	7,06%	28	8,97%	
	Total	**85**	**100,00%**	**312**	**100,00%**	
MMDS	Low (0-3)	16	19,51%	98	31,41%	0,0915
	Medium (4-6)	54	65,85%	181	58,01%	
	High (7-8)	12	14,63%	33	10,58%	
	Total	**82**	**100,00%**	**312**	**100,00%**	
Smoking status	Smoker	74	85,06%	194	61,98%	- 0,0001
	No smoker	13	14,94%	119	38,02%	
	Total	**87**	**100,00%**	**313**	**100,00%**	
Age (mean)		**52,87**		**68,36**		**<0,0001**

*p-value from chi-square test or Wilcoxon test as appropriate

Table 23. *Crude mean of M$_1$dG in gastric cancer population versus controls*

	Mean M$_1$dG	N	Standard Dev.	P-value*
Controls	2,1812	313	0,09	< 0,0001
Gastric cancer	3,4122	87	0,19	

*p value from Mann Whitney U test

Table 24. *Sex – and age- adjusted mean of M1dG in gastric cancer population and controls*

	Mean M$_1$dG	N	Standard Dev.	P-value*
Controls	2,125	313	0,11	< 0,0001
Gastric cancer	3,6138	87	0,24	

*p value from Dunnet test

Table 25. *Multiple adjusted** mean of M1dG in gastric cancer population versus controls*

	Mean M$_1$dG	N	Standard Dev.	P-value*
Controls	2,164	313	0,10	< 0,0001
Gastric cancer	3,6136	87	0,28	

- ****adjusted for sex, age, BMI, smoke, physical activity, education level, total caloric intake and the *MMDS***

*p value from Dunnet test

The comparison of the crude mean of M1dG in gastric cancer population sample versus healthy controls sample show significantly higher levels of M1dG adducts in cancer patients than in controls (p< 0,0001). This difference is is confirmed also when the M1dG mean is adjusted for sex and age and when the mean of M1dG is adjusted for multiple factors (sex, age, BMI, smoke, physical activity, education level, total caloric intake and the MMDS).

Table 26. *Crude Mean of M₁dG in gastric cancer cases and control by categories of MMDS*

		Controls	Crude Mean		Cases	Crude Mean	
			Mean	Standard		Mean	Standard
		N°	M1dG	Dev.	N°	M1dG	Dev.
MMDS	Low (0-3)	98	2,2959	0,1597	16	3,6250	0,5798
	Medium (4-6)	181	2,1643	0,1175	54	3,7227	0,3156
	High (7-8)	33	1,9560	0,2752	12	2,2833	0,6695
	Total	**312**			**82**		

Table 27. *Adjusted* Mean of M₁dG in gastric cancer cases and control by categories of MMDS.*

		Controls	*Adj Mean		Cases	*Adj. Mean	
			Mean	Standard			
		N°	M1dG	Dev.	N°	Mean M1dG	Standard Dev.
MMDS	Low (0-3)	98	2,3122	0,1611	16	3,5566	0,6098
	Medium (4-6)	181	2,1753	0,1180	54	3,6618	0,3258
	High (7-8)	33	1,8475	0,2798	12	2,6485	0,7184
	Total	**312**			**82**		

- *adjusted for sex, age, BMI, smoke, physical activity, education level

Then we conducted a comparison between the crude means of M1dG of the gastric cancer cases and those of the controls, in three categories of Modified Mediterranean Diet Score. The suggested inverse correlation between M₁dG and the Modified Mediterranean Diet Score (MMDS) found in the healthy control sample, is confirmed in the cancer gastric patients (the decrease of M1dG levels with the increasing of the MMDS has the same trend both in the gastric cancer sample population and in the control group).

The same comparison was conducted also between the adjusted means of M1dG of the cancer cases and those of the controls; the trend found was the same described above for the crude means of M1dG.

Breast Cancer

Selected individual characteristics of the 75 breast cancer patients are shown in Table 28 and compared to the controls.

The sample was composed of 75 women.

The population tended to be overweight; according to BMI classes, the 35,82% of the population sample was overweight and the 11,94% was obese. The proportion of current smokers was 86,67% and 13,33% of no smokers.

The physical activity level considering working, home and leisure time was moderately active for the 28,57% of the population sample and active only for the 5,71%. About the educational level, only the 8% had a degree.

Table 28. *Distribution of selected individual characteristics in breast cancer cases and controls*

Breast

Characteristics	Description	Cases		Controls		P-value*
		N°	%	N°	%	
Sex	M	0	0,00%	0	0,00%	
	F	75	100,00%	174	100,00%	
	Total	**75**	**100,00%**	**174**	**100,00%**	
BMI	Normal	35	52,24%	99	56,90%	0,7250
	Overweight	24	35,82%	53	30,46%	
	Obese	8	11,94%	22	12,64%	
	Total	**67**	**100,00%**	**174**	**100,00%**	
Education level	Degree	6	8,00%	49	28,16%	0,0384
	Other	69	92,00%	125	71,84%	
	Total	**75**	**100,00%**	**174**	**100,00%**	
PA ^	Inactive	14	20,00%	55	31,79%	0,2510
	Moderately inactive	32	45,71%	61	35,26%	
	Moderately active	20	28,57%	45	26,01%	
	Active	4	5,71%	12	6,94%	
	Total	**70**	**100,00%**	**173**	**100,00%**	
Smoking status	Smoker	65	86,67%	114	65,52%	0,0007
	No smoker	10	13,33%	60	34,48%	
	Total	**75**	**100,00%**	**174**	**100,00%**	
Menopause	No	20	26,67%	74	42,53%	0,0178
	Yes	55	73,33%	100	57,47%	
	Total	**75**	**100,00%**	**174**	**100,00%**	
Age (mean)		**n.a.**		**n.a.**		**n.a.**

*p-value from chi-square test or Wilcoxon test as appropriate

Table 29. *Crude mean of M_1dG in breast cancer population versus controls.*

	Crude Mean M1dG	N	Standard Dev.	P-value*
Controls	2,2149	174	0,11	0,0729
Breast cancer	2,5913	46	0,17	

*p value from Mann Whitney U test

Table 30. *Age- adjusted mean of M_1dG in breast cancer population and controls*

	Adj. Mean M1dG	N	Standard Dev.	P-value*
Controls	2,225	174	0,12	0,1213
Breast cancer	2,5678	46	0,18	

*p value from Dunnet test

The comparison of the crude mean of M_1dG in breast cancer population sample versus healthy controls sample show higher levels of M_1dG adducts in cancer patients than in controls, but the results is not significant (p=0729) . When the mean of M_1dG is adjusted for age, the difference between cancer cases and controls further decrease (p=0,1213).

Discussion

In the first part, the present study analyses the association between the most frequent MDA-DNA adduct (M_1dG), a biomarker of oxidative stress, and some potential environmental and individual factors in a general population. The potential factors are suggested from the review of the available literature.

To our knowledge, the observational studies or the intervention trials conducted on humans about MDA and its environmental and individual determinants are in a limited number (roughly one hundred). The majority of the studies is based on a limited size sample of people. There is not homogeneity between the studies (particularly for the use of different methods of detection for MDA, and for the statistical analyses, evaluating associations, performed with different programmes) These elements could at least partially explain the difficult to compare the studies reviewed and the inconsistence of the results that sometimes emerges.

Many studies evaluated the plasmatic levels of MDA, while a few studies are focused on MDA-DNA adducts. In fact, concerning M_1dG adducts we found only four observational studies conducted on humans and none intervention trials conducted on humans (Table 1). We can so assume that our study is very innovative in field of the MDA-DNA adducts. Besides, our sample size is higher than that of many of the reviewed studies (table 1); the collection of information from the population sample was accurate and conducted using standardized questionnaires and data elaboration settings already validated in epidemiological international studies (Masala 2003)

To select the environmental factors to test in our study, we assumed that they are the same for MDA and for its DNA adducts. This is a really plausible assumption, even if from a theoretical point of view, MDA and its DNA adducts are intrinsically different and not comparable at all. MDA is a damaging genotoxic chemical molecule, generated in oxidative stress conditions. MDA-DNA adducts represent mutagenic and carcinogenic biochemical entities, in which the initial DNA-damage have already happened and where the systems of genes repair did not manage to protect DNA from damage.

According to the literature review we have performed, the major environmental determinants that seem to be chiefly linked with increasing oxidative stress and consequently MDA levels, are:
- a diet rich in lipid, meat, fried food, dairy products
- smoke (active and passive smoking)
- alcohol assumption
- acute physical activity
- obesity
- environmental pollution
- job and life stress

Instead, the major environmental determinants that seem to chiefly allow the organism to maintain low levels of oxidative stress and consequently of MDA, are:
- a diet rich in fruit and vegetable, with high antioxidant power.
- constant physical activity

The literature studies analyzing the relation between foods and MDA did not lead to univocal results. First of all, there are food products (e.g. potatoes, red wine..) or nutrients (e.g, carboidrates, proteins, vitamin E..) for which the linkage with MDA is tested only in

one or at least two previous studies. The most frequent reported results are that the consumption of vegetables and fruit is linked to lower levels of MDA; instead, the consumption of dairy products, grilled or fried foods, is linked to higher levels of MDA. This last result did not emerge clearly from our review of the literature (observational studies or intervention trials conducted on humans are few about this kind of food products), but it is confirmed in animals and laboratory studies.

With regards to obesity, it is mainly found that it is a condition in which oxidative stress (including MDA) is higher that in normal weight people. It is interesting to remember that oxidative stress increases also according to the severity of insulin resistance which could be linked to the development of comorbidities, and that MDA levels might be partially reduced by dietary and exercise intervention.

Concerning the consumption of alcohol, markers of oxidative stress, especially MDA, are found higher, and antioxidant vitamin levels lower, in individuals who usually drink large amounts of alcohol in comparison to people who usually drink small amounts of alcohol. Moreover, patients having an hepatic alcohol related pathology (e.g. cirrhosis) show higher MDA levels than healthy controls.

About smoke, the major part of the studies revised in the literature about the linkage between MDA and smoke, underline the positive association between the increase of MDA levels and smoke. It was also observed that effects of passive exposure to tobacco are similar to those of active smoking in terms of oxidative stress and MDA, and that systemic and local MDA levels are increased by peridontitis (a common condition in smokers) in addition to the increase that smoke itself causes.

Concerning physical exercise, MDA levels are significantly higher and antioxidant levels significantly lower in active subjects than in sedentary subjects. This effect was found in individuals who usually practice a chronic and regular physical activity (particularly if an aerobic one). Instead, an acute episode of intermittent or anaerobic exercise induces immune suppression and increases the production of reactive oxygen species, causing oxidative stress (increasing MDA basal levels) both in trained and not-trained individuals.

According to our literature review, there is also a linkage between environmental pollution, work stress conditions and MDA increasing levels: the studies about this topic are few, but they lead to univocal conclusions. Anyway we don't have available data to examine this correlation in our study.

The main result emerging from our study on the 313 healthy subjects, is that oxidative stress and consequently M_1dG levels are lower in subjects having a greater adherence to Mediterranean style Diet (that is an higher Mediterranean Modified Diet Score-MMDS). Similar results were reported also by Azzini et al. (2011) and Kim et al (2011). Both these reviewed studies analyze MDA levels and not M_1dG levels (Azzini plasmatic MDA concentration and Kim urinary MDA concentration). The study of Azzini and colleagues are instead conducted on a population of 131 Italian subjects; in their analysis they used the Mediterranean Diet Score. The study of Kim and colleagues was conducted in Korea and it is one of the most numerous we found in literature during our research; they used an alternate Mediterrean Diet Score (aMDS). The interesting result that emerges from this section of our study is that when the individual components of the Mediterranean Diet, as well as individual antioxidants micronutrients, are considered alone, they don't produce a reduction in M_1dG levels in our statistical analyses. This result, that could initially appear contradictory, could be explained by the fact that the reciprocal interaction of the individual antioxidants micronutrients and of the components of the Mediterranean Diet, is the key element that produce a reduction in oxidative stress levels and in M_1dG levels.

The other result emerging from our study, is that oxidative stress and consequently MDA adducts levels are higher in female. To our knowledge, there are not consistent similar results in literature. Nair and colleagues (1997) showed that a very high intake of ω-6 PUFA, linoleic acid, in sunflower oil, but not of oleic acid in rape seed oil, increased the level of etheno-DNA adducts of 40-times in women (but not in men). These results suggested that the DNA adduction was attributable not only to the consumption of a linoleic acid-rich diet, but also to the gender-specificity. Sun and colleagues (2012) hypothesized that higher oxidative stress in female could be related to an interaction between ω-6 PUFA intake and estrogen catabolism.

About physical activity, our results suggest that a sedentary lifestyle is associated with higher levels of MDA adducts than an active or moderately active lifestyle. This result agree with the emerging results from literature review. A long-term physical activity is associated with preserved antioxidant defences and lower levels of MDA in comparison to sedentary subjects (Franzoni 2005). Contrary, an acute episode of intermittent or anaerobic exercise leads to oxidative stress increasing (including MDA) (Cardoso 2012).

Then, we found a borderline association with education level: an high educational level is associated with lower levels of M_1dG. In our review of the literature we were not able to find any paper evaluating the possible association between educational levels and MDA.

In the second part of our study, we analysed the association between M_1dG and cancer development. The studies available in literature mostly agree that MDA and M_1dG levels were higher in cancer cases than in healthy subjects. The sample population we studied was divided in three subgroups, according to the cancer they are affected (n=51, colon cancer; n=87 gastric cancer and n=75 breast cancer).

We focused the review of the literature about MDA and colon, gastric and breast cancer. Also in this case, M_1dG is measured infrequently; the main part of the studies investigate MDA plasmatic levels. To our knowledge, there are only two previous studies, measuring M_1dG (Peluso 2011, Wang 1996). Thus, also this section of our study is very innovative in this field.

With regards to the review performed, there are available in literature only a limited number of case control studies conducted on humans and also a limited number of laboratory studies where sample of neoplastic human tissue are compared to normal ones. Even if the majority of these studies is based on limited size of sample population/tissue, and even if different materials and methods are often used, there is homogeneity both in the aims and in the results emerging from the studies reviewed. Most of the reviewed studies agree that MDA levels and M_1dG increase in the presence of cancer.

The results of our study on 51 colon cancer patients and on 87 gastric cancer patients in comparison to the 313 healthy controls confirmed what expected from literature. We found that M_1dG levels are significantly higher both in gastric and in colon cancer population than in the control group. The difference is statistically significant (p<0,0001) and it is confirmed also after adjusting for several potential confounders (sex, age, BMI, smoke, physical activity, education level, total caloric intake and the *MMDS)*.

It is possible that an increment over the physiological level of M_1dG adduct generated during physiological processes can contribute to cancer development. Besides increased levels of M_1dG adduct in cancer cases can be reflective of impairments in the metabolic detoxification of oxidative by-products or in the DNA repair activity (Peluso 2011).

Moreover, these results, correlated to the biochemical pathways that lead to MDA formation, suggest that cancer condition is linked to a chronic inflammation status, possibly

induced by chronic sub-infections. There are many data in literature supporting this hypothesis.

First of all about gastric cancer, the role of *Helicobacter pylori* in gastric carcinogenesis has been established in epidemiological studies and also in animal models, as reported by Everett and colleagues. One possible mechanism for *H. pylori* mediated carcinogenesis is through induction of DNA damage and mutations as a result of increased activity of reactive oxygen species in the gastric mucosa. *H. pylori* infection of the gastric mucosa stimulates influx of polymorphonuclear leukocytes, leading to the generation of reactive oxygen and nitrogen species. Cell membranes, which are rich in polyunsaturated fatty acids, are readily attacked by these compounds, producing fatty acid radicals and lipid hydroperoxides, which can decompose in complex ways, yielding more radical species and a wide range of compounds, especially aldehydes (reactions with the lipid bilayer resulting in the accumulation of degradation products, such as MDA, has been shown to be present in increased concentrations in *H. pylori* gastritis). Of these aldehydes, MDA, which is also formed by the prostaglandin pathways, and 4-hydroxynonenal are the most common. These compounds can damage DNA directly by causing strand breaks, apurinic sites, or DNA adducts. Vitamin C is believed to be one of the major defenses against oxidative stress in the stomach and is concentrated several-fold from plasma to the gastric mucosa (vitamin C exists in two forms, dehydroascorbic acid and the potent antioxidant ascorbic acid). Concentrations of vitamin C are diminished in gastric juice of *H. pylori*-infected patients, in a process that may exacerbate oxidative damage .

Accumulation of MDA in *H. pylori*-infected gastric mucosa not only provides evidence of increased oxidative stress and lipid peroxidation, but also of its carcinogenic role. MDA can react with DNA to form adducts and cross-links and has been shown to be mutagenic in bacterial and mammalian systems and carcinogenic to rats. MDA induces a diverse spectrum of mutations, such as frameshift mutations and base pair substitutions in *Escherichia coli* . In humans, increased concentrations of lipid peroxidation products have been found in the serum of gastric cancer patients. The main DNA adduct is M_1dG, which has been shown in further *E.coli* studies to result in a mutation frequency, after correction for strand bias, of 18% (a 500-fold increase over unmodified DNA) (Fink 1997, Everett 2001).

In their study Everett and colleagues (2001) determined the effect of *H. pylori* and lipid peroxidation on levels of M_1dG in gastric biopsies and examined whether ascorbic acid protects against this process. They measured levels of M_1dG using the immunoslot- blot technique alongside mucosal MDA and plasma, mucosal, and gastric juice ascorbic acid and total vitamin C. They evaluated patients with normal and *H. pylori*-infected mucosa and followed *H. pylori* infected patients for up to 12 months after eradication of the microorganism. MDA levels resulted higher, gastric juice ascorbic acid was lower, and antral mucosal ascorbic acid was unchanged in *H. pylori* gastritis compared with normal mucosa. Even if levels of M_1dG did not differ between subjects with *H. pylori* gastritis and those with normal mucosa without *H. pylori* infection, multiple regression analysis revealed that M_1dG increased significantly with increasing levels of MDA. The innovative result is that M_1dG levels were unchanged 6 months and 12 months after successful eradication of *H.pylori,* meaning that M_1dG is probably an expression of that DNA damage induced by *H.pylori*, but it is not affected directly by it. (Everett 2001)

To our knowledge, there are not similar findings for colon cancer until now. Anyway many studies are moving in this direction.

Human gastrointestinal tract has an extremely complex and huge microbial colony, there are about 10^{13} to 10^{14} microbial organisms in the colon only. These microbes play an indispensable part of the body's normal physiological function by forming a symbiotic

relationship with the host, which helps the individual to survive (for example, digestion of food, nutritional energy conversion, production of short-chain fatty acids and essential vitamins etc.). (Huipeng 2013)

Besides, this bacterial population, also called the intestinal microbioma, plays an important role in the development of the gut immune system and resistance to colonization from pathogenic microorganisms.(Huipeng 2013, Behsen 2013)

The human gut microbiome consists of many different species of bacteria, some of which are not culturable and therefore not well known or characterized. Indeed, it has only been through the advent of deep sequencing, genomics, and metagenomics in the last decade that the complexity of the microbioma has been fully appreciated.

The distribution of the intestinal microbioma varies along three main locations in the digestive tract:

-the stomach, populated by 10^2 colony-forming units (cfu)/ml, including lactobacilli and streptococci;

- the ileum and distal ileum, populated by 10^2–10^3 cfu/mL of bacteria, including E. coli, Klebsiella, Enterococcus, and Bacteroides;

-the large intestine, which constitutes the largest microbial population of the body, with 10^{10}–10^{12} cfu/ mL.

Each individual organism presents a specific "bacterial fingerprint," which is influenced by a variety of factors including the maternal environment, host genotype, diet, and antibiotic treatment.

The composition of the microbioma differs from person to person; it clusters in three distinct groups, so called enterotypes. These human enterotypes are enriched in Bacteroides, Prevotella, or Ruminococcus and use different routes to generate energy from fermentable substrates available in the colon. The advent of germ-free mice gave rise to a better understanding of the impact the intestinal microbioma has on the host. Studies have shown that these mice exhibit a thin intestinal epithelium, loss of short-chain fatty acid production , and alterations to the immune system . Thus, probiotics are beneficial components of the microbioma and have been used for centuries because of the health benefits they confer to the host. (Behsen 2013)

Although microbioma is good and necessary for the host, different events such as age, body mass index, diets, antibiotic use, inflammatory bowel disease, and other chronic diseases such as heart disease, angiogenesis, endogenous adaptive immunity, metabolism, type I, type II diabetes, and kidney disease can affect the composition of the gut flora, resulting in the imbalance of microbial homeostasis, which can lead to colon cancer.

It was found that the intestinal flora not only produce lots of carcinogenic products in the metabolic process of intestinal contents but also participate in intestinal mucosal immune function, intestinal inflammation reaction, balance of mucosal cell proliferation, apoptosis, promoting DNA degeneration, and destruction of chromosome stability. Therefore, the relationship between intestinal flora and colon cancer is closely tied to each other.

In addition, it was showed that the flora structure of the fecal and mucosa is different from each other, and different parts of the mucosa flora structure are also slightly different. The flora of feces can only represent the flora structure within the gut cavity because the intestinal micro environment is different in the intestinal epithelial cell surface and gut cavity. (Huipeng 2013)

Several recent studies characterized the composition of the gut microbiome associated with patients with colorectal cancer. Using culture-independent approaches, these studies observed a significant shift in the composition of the gut microbiome in patients with colonrectal cancer compared to that in healthy controls. This phenomenon, referred to as dysbiosis, can be observed in both the luminal microbiome from feces and the mucosa-

associated microbiome from tumor biopsy specimens. Interestingly, each of these studies obtained conflicting results regarding the composition and structure of the CRC associated microbial community. Furthermore, there are no bacterial populations that have consistently been identified across each study that can be attributed to the development or presence of CRC. These data clearly show an association between abnormalities in the gut microbiome and CRC; however, the conflicting results point out the need for a mechanistic understanding of the role of the gut microbiome in this process. The combination of factors that could lead to dysbiosis is complex and not well understood, but recent evidence suggests that certain strains of *Bacteroides fragilis* and *Escherichia coli* can directly affect tumor development in the colon through the production of virulence factors (e.g., toxins and gene products). Furthermore, bacterial populations that produce the short-chain fatty acid butyrate have antitumor effects in the colon by promoting apoptosis of colonic cancer cells . The gut microbiome is also likely to contribute to CRC through the initiation of inflammation. The link between inflammation and cancer is well established, and patients with inflammatory bowel diseases, such as ulcerative colitis, are at a greater risk of developing CRC in their lifetime. In the case of ulcerative colitis, the risk for cancer is related to both the duration and severity of inflammation, with an increasing rate of 0.5 to 1% per year after the first decade. Chronic inflammation of the colon leads to the production of various inflammatory cytokines and reactive oxygen species that work in concert to generate a tumor microenvironment that promotes carcinogenesis. It has been suggested that this process is microbe driven, but it is unclear how the normally beneficial gut microbiome becomes inflammatory (Zackular 2013)

Some studies showed that mucosa-associated and mucosa-internalized *E. coli* were observed more often in colorectal cancer patients than in controls, supporting the central role of these bacteria in the development of colorectal cancer. A relationship between poor prognostic factors for colon cancer (TNM stage) and colonization of mucosa by *E. coli* was observed. Pathogenic cyclomodulin-positive *E. coli* strains were more prevalent on mucosa of patients with stages III/IV than those with stage I colon cancer. Mice infected with the *E.coli* strain 11G5 displayed a marked increase in the number of visible colonic polyps compared with controls.(Bonnet 2013)

The correlation of these data to the biochemical pathways that lead to MDA formation, supports the hypothesis that also colon cancer condition is linked to a chronic inflammation status, possibly induced by chronic sub-infections.

In our study we evaluated also the relation between M_1dG and the Mediterranean Diet in the subgroups of the gastric and colon cancer cases. We confirmed for the gastric cancer cases the same trend found in the general healthy population: oxidative stress and consequently M_1dG levels are lower in gastric cancer patients having a Mediterranean style Diet. This effect emerged both for crude and adjusted mean. The suggested inverse correlation between M_1dG and the Modified Mediterranean Diet Score (MMDS) found in the healthy control sample did not emerge instead in the colon cancer cases. However, in each category of MMDS the M_1dG values were higher among cases compared to controls. The same comparison was conducted also considering the adjusted means of M_1dG of the colon cancer cases and those of the controls, with similar results. This result could be explained by the fact that in a cancer population the basal level of MDA could be increased because of tumour biochemical themselves activated pathways.

The result of our analyses on the 75 breast cancer cases in comparison to the 313 healthy control show higher levels of M_1dG in breast cancer population than in general healthy population sample, but the difference is not statistically significant. The majority of the studies reviewed found MDA higher levels in breast cancer patients than in general

population. Anyway there are also some studies (more numerous than for colon and gastric cancer) that show a decrease in MDA levels in breast cancer population versus controls (from table 7 to table 12).

These contrasting results could be explained by the not yet fully understood role that estrogens could have in lipid peroxidation pathways, which could lead to a basal increase in lipid peroxidation in healthy pre-menopausal female, as previously explained about the general healthy population (Sun 2012). Thus, it is possible to hypothesize that these contrasting results could depend from different examined population (pre-menopausal versus post-menopausal women), but we don't have sufficient data to explore this correlation in our study, neither we were able to find any confirming data about this topic in the reviewed studies.

Another hypothesis to explain the difference in results between the gastrointestinal cancer examined and the breast cancer, could be that the gastrointestinal tract, because of its anatomy and function, is more exposed to environmental determinants (especially food products) than the breast tissue. This could be a possible hypothesis to explain the higher levels of M_1dG we found in the gastric and colon cancer population than in the breast cancer population in our study.

Moreover some reports suggest that lipid peroxidation may represent a protective mechanism in breast cancer through the induction of apoptosis, thereby maintaining the balance between breast epithelial cell growth and death, supporting the observation that lipid peroxidation in normal tissue or in tissue of benign breast disease is greater than in breast cancer (Mannello 2007). To explore better these correlation, we think that further studies are needed in this direction.

Summarizing, we firstly evaluated the lifestyle and dietary determinants of M_1dG adduct level in a representative sample of the general population of the city of Florence, composed of 313 clinically healthy subjects. The main result emerging from this part of the study is that oxidative stress and consequently M_1dG levels are lower in subjects having a greater adherence to Mediterranean style Diet.

Secondly, we conducted a case-control study, comparing M_1dG levels detected in subjects affected by colon, gastric or breast cancer (respectively 51, 87 and 75 subjects) and those detected in the representative sample of the general population (313 controls).We found that M_1dG levels are significantly higher both in gastric and in colon cancer cases than in the control group, while for the breast cancer cases this difference was not statistically significant.

In conclusion, our study suggests, as previously found by Peluso and colleagues (2011), that an increment over the background frequency of M_1dG adducts generated during normal physiological processes can contribute to development and progression of cancer. M_1dG adduct could be a useful tool for evaluating cancer risk in future clinical and epidemiological studies. An additional benefit will be their eventual application to chemoprevention of cancer (for example, reducing the levels of mutagenic M_1dG adduct could be a solution to slow the mutation rate of cancer cell

Acknowledgements

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