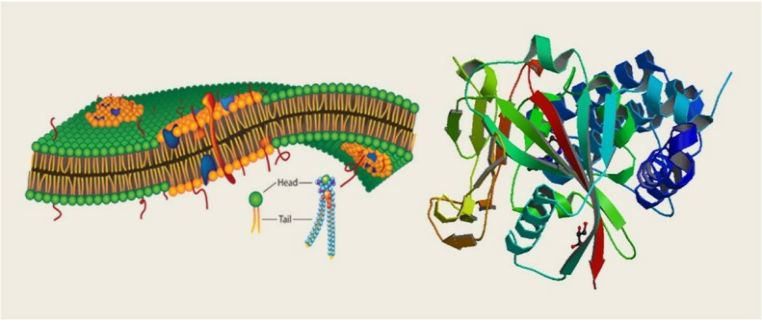
UNIVERSITA' DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN BIOCHIMICA E BIOLOGIA CELLULARE E MOLECOLARE XXVII CICLO

Anna Rita Bianchi

Lipidomic and poly(ADP-ribose) polymerase automodification analyses as laboratory tools to detect the physio-pathological state of the cell.



Academic Year 2013/2014

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Lipidomic and poly(ADP-ribose) polymerase automodification analyses as laboratory tools to detect the physio-pathological state of the cell.

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Riassunto

Il danno al DNA e la perossidazione lipidica delle membrane cellulari sono le principali e più studiate conseguenze dello stress ossidativo. I meccanismi alla base di questi processi deleteri sono in gran parte noti, e vi sono evidenze crescenti che essi possano essere modulati sia da un corretto stile di vita, inclusa l'alimentazione, che da modifiche post-traduzionali di proteine, come la poli(ADP-ribosil)azione.
La reazione di poli(ADP-ribosil)azione, catalizzata dalle poli(ADP-ribosio) polimerasi, e coinvolta in numerosi processi cellulari, è considerata tra le modifiche post-traduzionali con caratteristiche epigenetiche. Le poli(ADP-ribosio) polimerasi nucleari 1 e 2 sono attivate da tagli al DNA. L'iperattivazione si traduce in automodificazione delle stesse con lunghi polimeri di ADP-ribosio, che contribuiscono a reclutare il macchinario di riparazione dell'acido nucleico. Quanto più il danno al DNA è esteso, tanto maggiore sarà il livello di automodificazione delle poli(ADP-ribosio) polimerasi.
D'altra parte, i lipidi derivati dall'alimentazione vengono incorporati nelle membrane cellulari, possono essere molecole di segnalazione, dare origine a composti pro-infiammatori (ω6) ed anti-infiammatori (ω3), ed, in quanto costituenti delle membrane cellulari, sono buoni marcatori del loro squilibrio.
In questo lavoro di tesi sono riportati i risultati di uno studio condotto al fine di stabilire se la combinazione delle due diverse analisi, cioè la determinazione dei livelli di poli(ADP-ribosio) polimerasi modificata e l'analisi della composizione fosfolipidica della membrana eritrocitaria, possano rappresentare un valido strumento per valutare lo stato fisio-patologico della cellula, e correlarlo con lo stile di vita, la dieta o gli stati patologici.
Le due analisi sono state condotte, in doppio cieco, su 95 individui sottoposti ad endoscopia. I soggetti sono stati intervistati per raccogliere i dati anamnestici e clinici, se presenti. I linfociti e gli eritrociti sono stati preparati da sangue venoso per analizzare, rispettivamente, l'automodificazione della poli(ADP-ribosio) polimerasi e il contenuto dei fosfolipidi di membrana. I risultati sono stati poi valutati statisticamente.
La determinazione dell'automodificazione della poli(ADP-ribosio) polimerasi ha confermato che, per quelle patologie che si

accompagnano a livelli differenti di danno al DNA, l'automodificazione delle PARP correla con l'entità del danno, e rappresenta uno strumento per valutare lo stato clinico della malattia, in funzione del trattamento chirurgico/terapeutico applicato.
Dal profilo lipidico della membrana eritrocitaria dei vari soggetti, è emerso il livello di squilibrio dei lipidi, correlabile sia ad alimentazione/stile di vita che a stati infiammatori associati a patologie. Nella maggior parte dei casi presi in esame ciascuna delle analisi ha dimostrato di fornire, separatamente, informazioni sulle condizioni fisio-patologiche dei soggetti, e di ottenere indicazioni sia sullo squilibrio di membrana che di alterata funzionalità di specifiche vie del metabolismo del DNA e/o dei lipidi; in alcuni casi è risultata evidente una correlazione di entrambe le analisi, con specifiche patologie. Ciò suggerisce che la combinazione dei due test nella routine di laboratorio, potrebbe mettere in evidenza alterazioni a livello del nucleo e delle membrane, preliminari ad una diagnosi di una specifica patologia ottenibile poi dal quadro clinico.
Da questo lavoro è emerso che l'automodificazione della PARP e il profilo lipidomico della membrana definiscono possibili biomarcatori per un monitoraggio sensibile, non invasivo e di routine dello stato fisio-patologico delle cellule.

Summary

Membrane lipid peroxidation and DNA damage are the main and most studied consequences of free radicals. The mechanisms underlying these deleterious processes are mostly known and there are increasing evidence that they might be modulated by a correct quality of food intake and post-translational modification reactions (namely poly(ADP-ribosyl)ation), respectively.

Poly(ADP-ribosyl)ation, catalysed by poly(ADP-ribose) polymerases, affects many cellular events and has a recognized epigenetic role. Nuclear poly(ADP-ribose) polymerases 1 and 2 are hyperactivated by DNA strand breaks. They auto-modify with large polymers of ADP-ribose and recruit DNA repair proteins. The more the DNA strand breaks, the more poly(ADP-ribose) polymerase modifies itself.

On the other hand, dietary lipids can be signaling molecules, lead to pro-(ω6)/anti-(ω3) inflammatory compounds, and be included in biomembranes, good biomarkers of their unbalance.

Here, we report the results obtained from a study to establish whether the combination of two different analyses, i.e. detecting auto-modified poly(ADP-ribose) polymerase levels and analyzing erythrocyte membrane fatty acid composition, might help to monitor the physio-pathological state of the cell, and to correlate with lifestyle, diet or diseases. The two analyses were carried blindly on 95 subjects undergoing endoscopy. They were first interviewed, to collect anamnesis and clinical data, if present. Lymphocytes and erythrocytes were prepared from venous blood to assay poly(ADP-ribose) polymerase automodification and membrane fatty acid content, respectively. The results were statistically evaluated. The measure of poly(ADP-ribose) polymerase automodification confirmed that its levels correlate with DNA damage extent, within the same pathology, and allowed to monitor the clinical activity of the disease, depending on ongoing surgical/therapeutic treatment. Membrane fat profile was able to evidence unbalance of lipids linked to both diet/lifestyle and inflammatory states leading to diseases. In most analysed cases, each test demonstrated to evidence, separately, specific features of the physio-pathological state of the subject, i.e. membrane lipid unbalance, altered function(s) of specific pathways of DNA and/or lipid metabolism. In other cases, a correlation between both analyses

and specific pathologies emerged. This finding suggested that in the laboratory routine, combining the two tests might be a preliminary step to investigate the occurrence of a given disease, before confirming the diagnosis with other specific clinical analyses.
Both PARP automodification and membrane lipidomic profile provide possible biomarkers for sensible, non-invasive and routine monitoring.

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1. Introduction

The epigenome is an essential mechanism for the regulation of the genome that depends on modifications of DNA and histones but does not involve any change of the DNA sequence (Jablonka & Raz, 2009). Epigenetic changes have been observed in response to environmental influence (Reik, 2007). Stress, diet, behaviour, exposure to toxicants, and other factors, activate chemical switches that regulate gene expression. Diet is one of the most studied environmental factors in epigenetic changes (Waterland & Jirtle, 2003). There are increasing evidence that a correct quality of food intake and post-translational modification reactions (namely poly(ADP-ribosyl)ation) might modulate deleterious processes as membrane lipid peroxidation and DNA damage respectively.

1.1 Poly(ADP-ribosyl)ation and DNA damage response

Poly(ADP-ribosyl)ation is a post-translational modification of proteins catalysed by poly(ADP-ribose) polymerases, PARPs. PARPs use β-NAD^+ as a substrate to synthesize and transfer ADP-ribose (ADPR) units onto specific amino acid residues of target proteins (Hassa *et al*., 2006).

The acceptor proteins are covalently modified by ADP-ribose polymers (PAR) up to several hundred ADP-ribose residues with branching and large negative charges (Bai & Cantó, 2012). PAR can be rapidly degraded *in vivo* by poly(ADP-ribose) glycohydrolase (PARG), an enzyme that possesses both endo- and exoglycosidic activity and is the only protein known to catalyse the hydrolysis of ADP-ribose polymers to free ADPR, Figure 1 (Davidovic *et al.*, 2001).

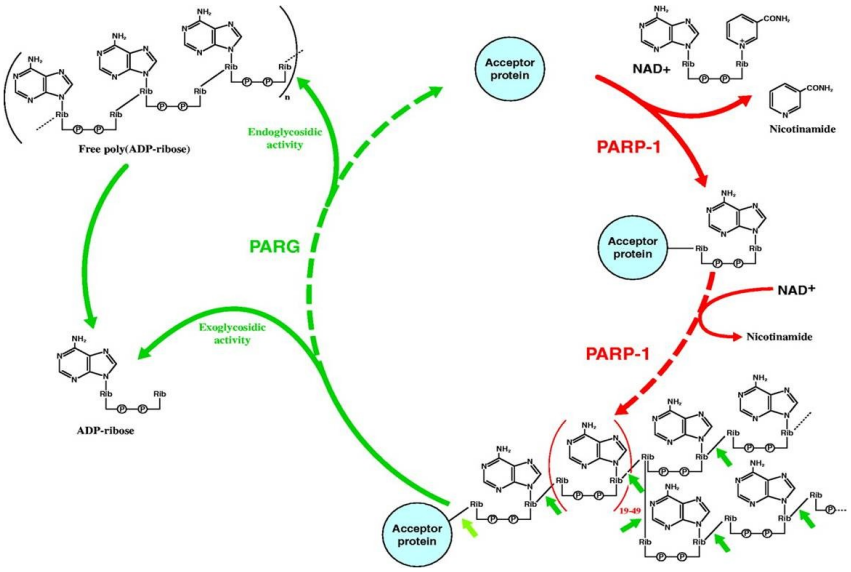

Figure 1: **Metabolism of poly(ADP-ribose)**.
Poly(ADP-ribose) polymerase 1 (PARP1) hydrolyzes NAD^+ to polymerize ADP-ribose units on substrate proteins, with the concomitant release of nicotinamide. PARP1 catalyzes the three steps of poly(ADP-ribose) anabolism (red part of the cycle): (1) mono(ADP-ribosyl)ation of the acceptor protein substrate; (2) elongation; and (3) branching of the poly(ADP-ribose) chain. PARG (green side of the cycle) cleaves the glycosidic bonds between ADP-ribose units both at the ends and within the poly(ADP-ribose) chains, respectively; the cleavage sites are shown by dark green arrows (Rouleau *et al.*, 2004).

In the human genome 18 different genes have been identified that all encode PARP family members, Figure 2 (Amé *et al.*, 2004). Not all PARP family members are enzymatically active, and some may function as mono(ADP-ribosyl) transferases rather than PARPs (Fabrizio *et al.*, 2015; Grimaldi *et al.*, 2015). As a consequence, Hottinger *et al.* (2010) proposed a new nomenclature describing PARPs more accurately as (ADP-ribosyl)transferases, ARTs, Figure 2 (Ryu *et al.*, 2015).

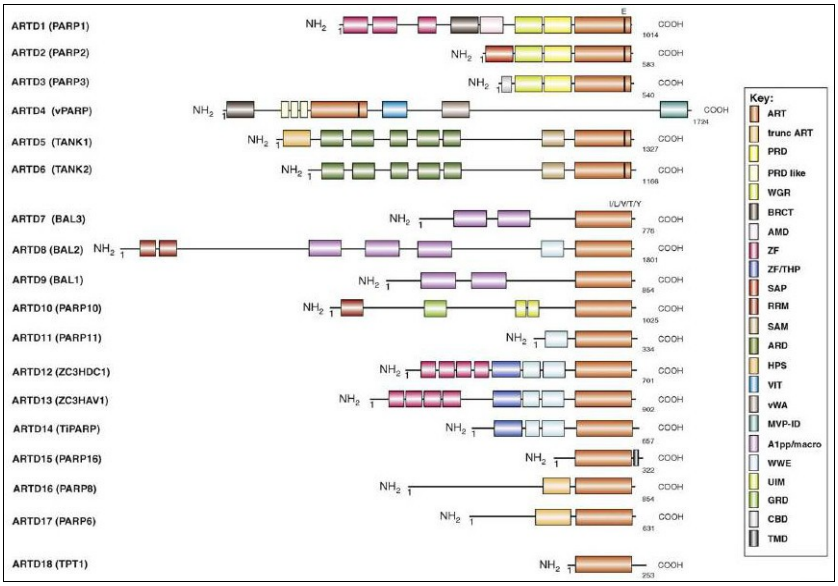

Figure 2: **Functional domains of the human ARTD (PARP) family.**
The most relevant are: **ART**, is the catalytic core required for basal ART activity. PARP regulatory domain (**PRD**) might be involved in regulation of the PARP-branching activity. **WGR**, characterized by the conserved central motif (W-G-R), is also found in a variety of a polyA polymerase and in protein of unknown function. **BRCT** domain (BRCA 1 carboxy-terminal domain) is found within many DNA damage repair and cell cycle checkpoint protein. **Macro** domain can serve as ADPR or 0-acetyl(ADP-ribose) binding module. **ZF**: zinc finger domains, recognizing DNA breaks (Hottinger *et al.*, 2010).

PARPs have key functions in a wide range of biological structures and processes (Li & Chen, 2014).
The most representative enzymes of the PARP family are poly(ADP-ribose) polymerase 1, PARP1 (113 kDa), the funding member, and poly(ADP-ribose) polymerase 2, PARP2, 62 kDa, Figure 3. Both are highly conserved proteins, ubiquitously expressed in mammalian tissues and with predominant nuclear localization (Bürkle, 2005). They are involved in several processes, as regulation of chromatin structure and transcription, cell proliferation and, as recently reported, in signaling and energy metabolism (Krishnakumar & Kraus, 2010; Yelamos *et al.*, 2011; Kraus & Hottinger, 2013; Golia *et al.*, 2015). Furthermore, PARP1 and PARP2 are involved in the first line of genome defense being efficient DNA-break sensors and signaling

molecules as a part of a survival program (Virág & Szabó, 2002; Beck *et al.*, 2014).
In human PARP1 the DNA binding domain (DBD) contains three zinc fingers to recognize DNA breaks; the central automodification domain (Domain E) has a motif (BRCT) for protein-protein interactions, and numerous glutamate residues for auto-poly(ADP-ribosyl)ation (auto-PARylation). In human PARP2 the highly basic DBD has not zinc fingers and is structurally different from that of PARP1 likely explaining the differences in the recognized DNA structures. PARP2 binds less efficiently to DNA single-strand breaks (SSB) but recognizes gaps and flap structures. Domain E (homologous to the E domain of PARP1), acts both as an automodification domain and as homodimerization interface (Schreiber *et al.*, 2006; Steffen *et al.*, 2013).

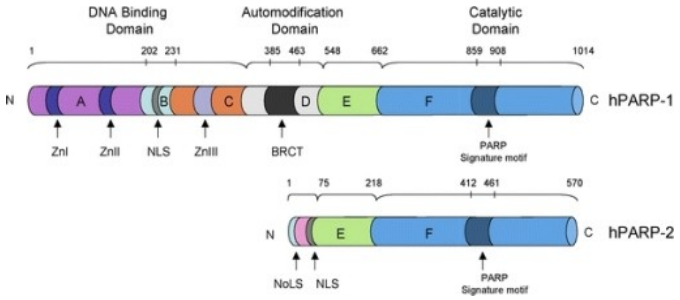

Figure 3: **The domain structure of human PARP1 and PARP2.**
Domains (domains A-F) correspond to DNA-binding, automodification and catalytic regions. The PARP signature sequence (in dark blue within the F catalytic domain) is the most conserved between PARPs. ZnI and ZnII: Zinc-finger motifs. ZnIII: Zinc ribbon domain. NLS nuclear localization signal; BRCT BRCA1 carboxy terminus; NoLS nucleolar localization signal (Mégnin-Chanet *et al.*, 2010).

Interestingly PARP1 and PARP2 are themselves PAR acceptors, undergoing automodification (PAR-PARP) and acting as autoregulatory negative feedback (Krishnakumar & Kraus, 2010).
PARP itself has been found to be the main acceptor of ADP-ribose polymers, both *in vitro* and *in vivo* (D'Amours *et al.*, 1999). The enzyme acts as a dimer, and each monomer builds long and branched (up to 200 residues) ADP-ribose polymers (Amé *et al.*, 2004). Once

the enzyme is activated, along with automodification, it can start a series of heteromodification reactions that modulate the functions of chromatin proteins (Muthurajan *et al.*, 2014). However, besides binding to DNA, PARP interacts with other proteins and it has been shown that such interactions may depend on its automodification state (Griesenbeck *et al.*, 1997).

Strong evidence is provided for a specific relationship between DNA damage and acute depression of cellular NAD^+ levels and that PARP1 can influence metabolic homeostasis (Surjana *et al.*, 2010; Faraone-Mennella, 2015). PARP1 is a DNA damage sensor that can be activated in response to DNA damage by various patho-physiological conditions, including oxidative stress and inflammatory injury. The NAD^+-consuming process is a consequence of PARP1 hyperactivation (Luo & Kraus, 2012; Kim *et al.*, 2014).

In the case of mild DNA damage, poly(ADP-ribosyl)ation facilitates DNA repair and thus survival. In the case of extensive one PARP1 leads to depletion of NAD^+ pools in cells upon hyperactivation, and attenuates NAD^+ dependent ATP production, resulting in cell death (Jagtap & Szabó, 2005). When DNA damage is irreparable, PARP1 activation induces the energy-dependent apoptotic process, by preventing ATP depletion and DNA repair through caspase-mediated PARP1 cleavage (Rolli *et al.*, 2000; Surjana *et al.*, 2010). In the case where PARP uses substantial amounts of its substrate, cells consume ATP in an attempt to replenish NAD^+, this leads to a cellular energy crisis, which precipitates necrotic cell death (Virág & Szabó, 2002).

During the apoptotic process, PARP is inactivated by caspase 3 by a single proteolytic cleavage of the 113 kDa active enzyme that produces an 89 kDa C-terminal fragment, with a reduced catalytic activity, and a 24 kDa N-terminal peptide, which retains the DNA binding domains, Figure 4 (Soldani & Scovassi, 2002; Aredia & Scovassi, 2014).

PARP2 has been shown to be cleaved by caspase 8. PARP2 cleavage, similar to PARP1 cleavage, separates the DNA binding end-catalytic domains and inactivates the enzyme (Benchoua *et al.*, 2002).

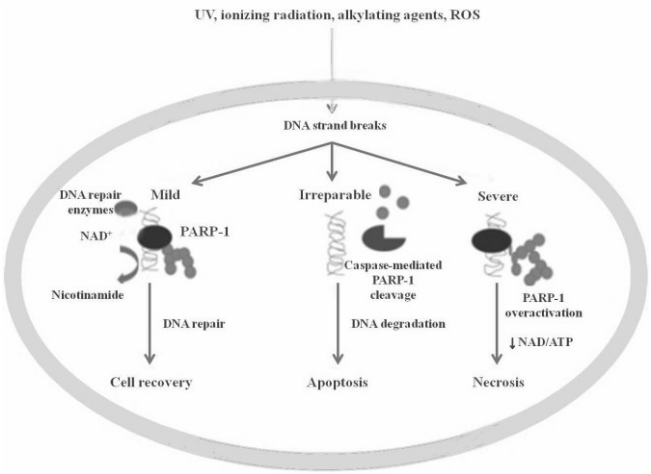

Figure 4: **PARP1 and DNA damage** (Surjana *et al.*, 2010).

Interestingly, PARP1 over-expression has been observed in various human tumour types and frequently correlated with a poor outcome, while the expression of PARP2 in cancer samples and its linkage with evolution of the disease is not completely known (Yelamos *et al.*, 2011). For instance, increased expression of PARP1 has been reported in the early stage of colorectal carcinogenesis (Nosho *et al.*, 2006), intestinal adenomas of patients with familial adenomatous polyposis (Idogawa *et al.*, 2005), hepatocellular carcinoma (Quiles-Perez *et al.*, 2010), endometrial hyperplasia (Ghabreau *et al.*, 2004), breast, uterine, lung, and ovarian cancers (Ossovskaya *et al.*, 2010).
Moreover, poly(ADP-ribosyl)ation has a central role in inflammation. PARPs have been implicated in the regulation of immune functions by regulating the expression of inflammatory mediators such as cytokines, chemokines, adhesion molecules, nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2). Free radicals may cause DNA damage and activate PARP1 and PARP2 (Heller *et al.*, 1994; Hegedűs & Virág, 2014). In case of severe DNA damage, PARP over-activation may cause cell dysfunction which may contribute to inflammation. The association between inflammatory pathologies and PARP2 (Szántó *et al.*, 2012), PARP3 (Phulwani & Kielian, 2008), tankyrases (Levaot *et al.*, 2011), or PARP14 (Mehrotra *et al.*, 2011, 2013) has already been shown (Bai & Virág, 2012).

On the basis of these observations, the role of PARP as biomarker of DNA damage is widely demonstrated; PARP automodification is recognized a direct signal of DNA damage. Therefore, it is conceivable that its activity levels/expression in the cell nucleus can help in defining the physio-pathological state of the cell, offering a number of opportunities for therapeutic intervention in both cancer and other disease states (Morales *et al.*, 2014).

1.2 The lipidomics of cell membrane

Lipidomics describes and quantitatively analyses the full complement of lipids, in the human body (body fluids, cells, tissues), and integrates these data with knowledge of their protein targets, i.e. the metabolic enzymes and transporters, and of the relevant genes and the regulatory aspects of these physiological systems. Lipids are energy biosources. Many intermediates of lipid metabolism serve a second function as primary or secondary messengers, under strict regulatory control. A variety of lipids serves to anchor proteins and glycans to membrane surfaces, thereby affecting and regulating their functions. Finally, lipids are the building blocks of cell membranes, providing these membranes with their physical characteristics. An understanding of cell membranes is only possible with a comprehensive understanding of their lipid constituents (Smilowitz *et al.*, 2013).

The cell membrane is highly organized and extremely important for a correct performance of the functions of the cell; it is a sensor that changes and adapts continuously to metabolic stimuli and the external environment (diet, stress, physical and chemical agents) and its properties are regulated by fatty acid composition. The membrane FA asset, i.e., saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA), present in the phospholipids is characteristic of each tissue. A natural adaptation response is active and the appropriate changes of the FA microenvironment ensure the best functioning of membrane proteins, receptors, pumps and signals in tissues, according to environmental and metabolic needs (Chatgilialoglu & Ferreri, 2012).

Fatty acids can be classified into saturated (SFA) and unsaturated, depending on the presence or absence of double bonds. The unsaturated are distinguished in monounsaturated fatty acids (MUFA), characterized by a single double bond, and polyunsaturated fatty acids (PUFA) that have two or more double bonds. The presence of double

bonds affects the structural properties of the membranes by increasing their fluidity and permeability, establishing the correct hormone-receptor binding and membrane transporter functioning (Russo, 2009).
Starting from the methyl group, defined as omega-one position, PUFAs are further subdivided in omega 3 (ω3) and omega 6 (ω6) fatty acids, depending on the position of the C atom were the first double bond occurs.
In the human body α-linolenic acid, the precursor of the omega 3 series, and linoleic acid, the precursor of omega 6 series, cannot be synthesized. Therefore, they are defined essential fatty acids (EFA); a correct diet will allow the intake of α-linolenic acid, and linoleic acid.
The catalytic action of enzymes named desaturase and elongase, allows the conversion of these precursors to different intermediate and final products, which are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for the omega 3 fatty acids, dihomogamma-linolenic acid (DGLA) and arachidonic acid (AA) for the omega 6 series, Figure 5 (Nakamura & Nara, 2004).
EPA and DHA give rise to a series of products with anti-inflammatory effects, generally, with preventive effect against various diseases. In contrast, high concentrations of ω6 fatty acids, expecially AA, lead to an increase of pro-inflammatory stimuli (Caramia, 2008).
The importance of the ω6/ω3 ratio has been evoked not only in the inflammatory and cardiovascular pathologies, but also in cancer and autoimmune diseases (Russo, 2009; Sheppard & Cheatham, 2013).
EFAs, being precursors of AA, play a crucial role in the synthesis of prostaglandins (PGs) by a cyclooxygenase (COX) enzyme. The alteration of this process would seriously affect many normal metabolic processes. Two COX isoforms have been identified, COX1 enzyme is expressed constitutively in most cells and tissues under either physiological or pathological conditions, COX2 is normally absent from most cells but highly inducible in certain cells in response to inflammatory stimuli resulting in enhanced PG release. It is suggested that consumption of a diet enriched in ω3 PUFA (specifically EPA and DHA) and inhibition of COX2 may confer cardioprotective effects and provide a significant mechanism for the prevention and treatment of human cancers (Tapiero *et al.*, 2002).

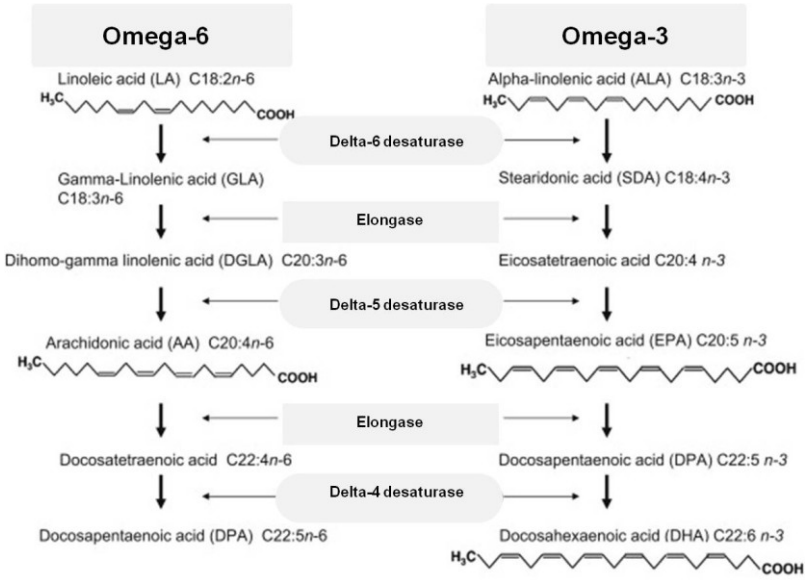

Figure 5: **Biosynthesis of omega 6 and omega 3 PUFAs.**

The synthesis of AA-derived eicosanoids is influenced by the concentration of DGLA. The excess of DGLA competes with AA for COX and lipoxygenase (LOX), inhibiting the production of AA-derived eicosanoids and driving the synthesis of DGLA-dependent prostaglandins (Dooper *et al.*, 2003).

Increased ω6 and reduced ω3 long-chain PUFA (LC-PUFA) intake in diets may contribute to the increased prevalence of allergic diseases. The ω6 LC-PUFA AA enhanced pro-inflammatory mediator production by effector cells in allergy (mast cells), while the ω3 LC-PUFA EPA as well as DHA more effectively suppressed reactive oxygen species (ROS) generation and interleukin-4 (IL-4) and interleukin-13 (IL-13) release. This suggests that dietary supplementation with EPA and/or DHA may alter the mast cells phenotype, contributing to a reduced susceptibility to develop and sustain allergic disease (van den Elsen *et al.*, 2013). Also, oral supplementation of the PUFA AA and DHA together with galactooligosaccharide and polydextrose (GOS and PDX) ameliorates the disturbed epidermal skin barrier of allergen-induced dermatitis due to a reduction in the local inflammatory immune response; the

normalization of the skin status is most likely dependent on direct action of DHA/AA or their bioactive metabolites on keratinocytes and mast cells (Weise *et al.*, 2013).
Recent studies have shown that ω3 polyunsaturated fatty acids (PUFA) and statins have antiviral properties *in vitro* (Sheridan *et al.*, 2014). AA, DHA and EPA inhibit hepatitis C virus (HCV) replication *in vitro* using the HCV RNA replicon system. ω3 PUFAs may inhibit HCV replication in vitro by a mechanism that is independent of their ability to suppress lipogenic gene expression (Kapadia & Chisari, 2005).
Lipids and phospholipids are the most susceptible molecules to stress conditions, including lipid remodelling and ROS attack, with consequent changes of fatty acids contents.
It is worth mentioning that in humans the unsaturated fatty acids (MUFA and PUFA) have the unsaturations (double bonds) in the *cis* configuration, displaying a bent structure that is very important for the fluidity of membranes.
The radical stress can induce an alteration in the normal *cis* configuration, leading to the formation of *trans* isomers, with a straight aliphatic chain (Figure 6). This different geometric disposition causes a greater membrane packing by altering its biological and biophysical properties. The consequences can affect metabolism and pathological processes.

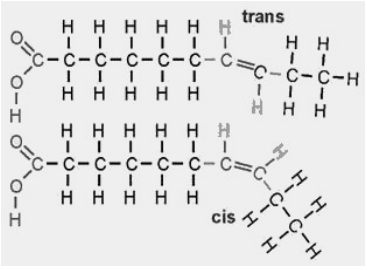

Figure 6: ***cis* and *trans* fatty acid isomers.**

Therefore the knowledge of the membrane status can be very important for the evaluation of the overall metabolic functioning (Ferreri & Chatgilialoglu, 2012).

In this context, lipidomics study membrane lipids, comparing their quality and quantity in relation to physiological and pathological conditions of the membranes. Membrane fatty acid composition is typical for each tissue and particularly the erythrocyte membrane has a peculiar percentage distribution of fatty acids and its lipid composition reflects the constant behavior in diet and the status of the subject.
The important role of changes to the membrane structure and corresponding physical-chemical properties are well established (van Ginkel & Sevanian, 1994).
Changes in the packing of membrane components, molecular redistributions, increased permeability, lateral fluidity, occur upon membrane reorganizations following oxidation of lipids by ROS, by influencing the conformations and thus the functions of selected membrane proteins, and leading to pathological conditions (Simopoulos, 2002; Baritaki *et al.*, 2007; Calder, 2009).

1.3 Scientific hypothesis and aim of the work

PARP automodification and membrane fatty acid composition are molecular aspects which can be taken as the mirror of the healthy/pathological state of the cell. In fact they both have been independently used in the last decade by several authors to look at membrane status and DNA damage in the cell.

Our working hypothesis is that combining the measure of lymphocyte PARP automodification (PAR-PARP levels) with erythrocyte membrane lipid profile (fat profile), allows to draw either prognostic or diagnostic clinical picture to support therapeutic and/or nutraceutical approaches. Despite many evidence available in literature suggest that increased oxidative stress and genomic instability can be monitored as early signals for prevention, these molecular aspects have not been examined in epidemiological and clinical settings yet.

Aim of the present research was to study the possibility that the two methods applied together in the laboratory routine could represent non invasive tests to determine the physio-pathology of cells by using venous blood as source of erythrocytes (for membrane fatty acid profile) and lymphocytes (for detection of automodified PARP levels). The analyses were performed on a cohort of subjects recruited at the Department of Clinical and Surgical Medicine (University of Naples "Federico II") for blood samples and clinical profiles.

The research was developed in three phases. First the samples underwent blindly to both tests. In the second phase, the results were compared with anamnestic and clinical data, and tabulated. Lastly, all data underwent statistical analyses. The initial statistic analysis was done after 70 individuals were examined. This number further increased up to 95 subjects. Two approaches were followed: first, a series of statistical analyses were performed grouping the subjects on the basis of their body mass indexes (BMI); thereafter the same people, implemented with new samples, were considered statistically on the basis of their pathologies. The latter results were used to find out any correlation between the two tests and the occurring diseases.

The rationale of BMI analysis (first approach) arose from the observation that overweight and obesity have recently reached vast proportions worldwide, representing one of the main historical metabolic risk factors. The onset of these conditions of excess weight has remarkable effects on health status with an increased risk of

inflammatory and cardiovascular diseases and some cancers. In this context, the first evaluation criterion was the comparison of the results of the analyses with both anthropometric measures (body mass index) and any clinical state of subjects undergoing the survey.
Second, a statistical analysis followed to evaluate the possible correlation of PARP automodification values with the clinical data and lipid profiles of the subjects, with the aim to study whether these analyses can have a predictable and/or early diagnostic meaning.

2. Materials and Methods

The volunteers (95) object of this survey, ageing 20-80 years, were recruited at the Department of Clinical Medicine and Surgery for interview, blood collection and routine diagnostic and clinical analyses. They underwent endoscopies either to confirm or to get diagnosis of intestinal pathologies. They all signed an informed consent for anonimous treatment of their data, according to the privacy rules.

Blood (8 ml) was collected by venipuncture in the presence of EDTA and aliquoted. One aliquot (4 ml) was used within few hours to prepare lymphocyte fraction. The second aliquot was used for erythrocyte membrane fatty acid composition.

2.1 Lymphocyte preparation and lysis

All operations were performed at 4°C. 4 blood aliquots (1 ml) of each sample were separately used to prepare lymphocytes, after counting the cells per ml of blood by hematic cell counter. In this way taking into account a comparable loss of lymphocytes during preparation under the same conditions for all samples, the results were referred to the initial number of cells/ml.

Lymphocytes were prepared according to GE Healthcare protocol, provided with Ficoll. Briefly each blood aliquot (1 ml) was layered on a Ficoll-Hypaque (GE Healthcare) cushion (1:0.7, v/v) and centrifuged at 2,500 rpm for 10 minutes. Because of their lower density, the lymphocytes were found at the interface between the plasma and the Ficoll-Paque PLUS with other slowly sedimenting particles (platelets and monocytes). The lymphocytes were recovered from the interface and subjected to short washing steps to remove any platelets, Ficoll-Paque PLUS and plasma. Crude lymphocyte fraction was washed twice with 0.9% NaCl, followed by 10 min centrifugation at 1,500 rpm. Pelleted pure lymphocytes were suspended in 0.9% NaCl (100 µl/blood ml). Few microliters of the suspension were used for counting pure lymphocytes. In general a 20% loss was measured. Pure lymphocytes were often used as freshly prepared fraction or stored at -80°C until used. Cells from 1 ml blood were lysed by suspension in lysis buffer (300 µl; 10mM Tris-HCl pH 7.5, 1% Nonidet P40, 2mM Spermidine-HCl, 10mM Na_2EDTA, protease inhibitor cocktail 2µg/ml (Sigma), 1mM Phenyl Methyl Sulphonyl

Fluoride, PMSF) and incubation for 30 minutes at 4°C. The whole lysate was further analysed. Protein content was determined by Bradford's reagent (Bio Rad) according to the provided instructions.
In order to determine whether increased PAR-PARP levels corresponded to damaged DNA, few cells from three normal and three PAR-PARP altered samples underwent to comet assay according to Atorino *et al.* (2001) and D'Onofrio *et al.* (2011). This technique is routinely performed on single cells by a research group at the Department of Biology.

2.2 SDS-PAGE and immunoblotting

2.2.1 Immunochemical analysis

Immunochemical analysis were performed blindly on blood samples collected from both healthy people and patients with different pathologies, according to Faraone-Mennella *et al.* (2010). Polyclonal anti-PARP1 catalytic site antibodies (H-250, Santa Cruz) were used to evidence PARPs.
The highly conserved catalytic domain allowed to recognize any PARP with these antibodies.

Alkali incubation of automodified PARP (PAR-PARP) was carried on in 10mM Tris-HCl pH 9.5, for three hours at room temperature, optimal conditions for complete removal of the polymer from the acceptor protein.

2.2.2 Densitometric analyses

Automodified PARP was quantified by densitometric analyses of immunobands with a Chemidoc apparatus (Bio Rad) and expressed as optical density (OD, i.e. intensity of a band)/mm^2.
To measure the densitometric value a rectangle was drawn encircling the band to quantify. The area of the rectangle was always constant. Therefore the variable parameter was the intensity of the band. Even if the Quantity One program software automatically subtracted a mean value of the densities measured all over the filter (blank), a background value was manually subtracted. This manual blank was measured by using the above described rectangle on a zone of the filter without bands. In addition, the results were normalized by performing densitometric measures of images with the same time of

development and chemiluminescent capture signals, as determined by preliminary analyses. In this way the results could be easily compared for all experiments.

2.3 Membrane fatty acid determination

An aliquot (4 ml) of each blood sample was used for the fatty acid analysis of erythrocyte membrane performed by the procedure described in Ghezzo *et al.* (2013).

This procedure is effected by an automatism set up at Lipinutragen (Ferreri, 2008), a spin-off company of the National Council of Research (CNR) in Bologna (Italy), connected with the calculation of the membrane unbalance index.

2.4 Body mass index

Body mass index (BMI) measured body height and weight of adult men and women to screen for weight categories that may lead to health problems, classified as under-, normal-, over-weight and obese. It was calculated as the ratio between weight in kilograms divided by the square of the height in meters (kg/m^2). The antropometric data, known only for 84 of 95 subjects, allowed to calculate their body mass indexes (BMI). On this basis three groups were identified: normal weight (19<BMI<25); overweight (25<BMI<30); obese (BMI>30).

2.5 Statistical analyses

Statistical analyses were performed using Microsoft Excel and Graph Pad Prism Programs.

The data were statistically analyzed with both parametric and non parametric tests.

The applied parametric procedures, as ANalysis Of VAriance, ANOVA, rely on assumptions about the shape of the distribution (i.e., assume a normal distribution) in the underlying population and about the form or parameters (i.e., means and standard deviations) of the assumed distribution (Conover, 1980; Rosner, 2000). ANOVA allowed to identify whether there are significant differences between the means of three or more independent (unrelated) groups. In the context of medical statistics, Receiver Operating Characteristic (ROC) curve, based on a parametric approach, has become the method of choice for quantification of accuracy of medical diagnostics tests

(Erdreich & Lee, 1981; Henderson, 1993). It is widely applied in measuring discriminatory ability of diagnostic or prognostic tests.
For multivariate complex problems, when the distribution model is not known, and the sample size is less than the number of variables, it is useful to change from a parametric approach to a non parametric one. Non parametric tests are the Chi square (χ^2 test), based on the frequencies, and the Mann Whitney U-test based on sorting of the information. Chi-square is used to compare observed data with those expected according to a specific hypothesis. The Mann Whitney U-test allows to know whether two independent samples of observations are drawn from the same or identical distributions.
Spearman's Correlation was applied too.
Details of ROC curve, Mann Whitney U-test, Spearman's rank correlation coefficient are reported below.

2.5.1 Receiver Operating Characteristic (ROC) curves

ROC curves are constructed by computing and comparing the sensitivity (y-axis) and specificity (x-axis= 1-specificity) of increasing numbers of clinical findings. High sensitivity corresponds to high negative predictive value; high specificity corresponds to high positive predictive value.
An important measure of the accuracy of the clinical test is the area under the ROC curve (AUC) that measures discrimination, i.e the ability of the test to classify correctly those with and without the disease (Zweig & Campbell, 1993). If this area is equal to 1.0 then the ROC curve consists of two straight lines, one vertical from 0.0 to 0.1 and one horizontal from 0.1 to 1.1. A ROC curve that is the diagonal line from 0.0 to 1.0 corresponds to a test that cannot discriminate between normal and abnormal subjects. Area for this line is 0.5. The greater the area under the curve, the better is the discriminatory power of the test. Significant ROC curve areas are typically between 0.5 and 1.0 (Swets, 1988).

2.5.2 Mann Whitney U-test

The results of the ROC curves were validated with the non parametric Mann Whitney U-test, that verifies the significance of the differences among medians (Soliani, 2001). The U-test is based on the precedences' number. Considered two groups (A and B), a correct count is how many times each data of A group is preceded by data of

B group. The lowest amount of precedences is the U value. If the two groups belong to different populations, the median of a group is considerably less than the other, and the value of U will tend to 0.
The test is significant ($\alpha \leq 0.05/0.01$) when the calculated value of U is less than or equal to the tabulated value, considering the size of groups (n_1 and n_2), Table 1.

Table 1: **Critical values of the Mann Whitney U-test**.

n_2	α	n_1																	
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
3	.05	0	0	1	2	2	3	4	4	5	5	6	7	7	8	9	9	10	11
	.01	--	0	0	0	0	0	1	1	1	2	2	2	3	3	4	4	4	5
4	.05	0	1	2	3	4	5	6	7	8	9	10	11	12	14	15	16	17	18
	.01	--	--	0	1	1	2	3	3	4	5	5	6	7	7	8	9	9	10
5	.05	1	2	4	5	6	8	9	11	12	13	15	16	18	19	20	22	23	25
	.01	--	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
6	.05	2	3	5	7	8	10	12	14	16	17	19	21	23	25	26	28	30	32
	.01	--	1	2	3	4	6	7	8	9	11	12	13	15	16	18	19	20	22
7	.05	2	4	6	8	11	13	15	17	19	21	24	26	28	30	33	35	37	39
	.01	0	1	3	4	6	7	9	11	12	14	16	17	19	21	23	24	26	28
8	.05	3	5	8	10	13	15	18	20	23	26	28	31	33	36	39	41	44	47
	.01	0	2	4	6	7	9	11	13	15	17	20	22	24	26	28	30	32	34
9	.05	4	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54
	.01	1	3	5	7	9	11	14	16	18	21	23	26	28	31	33	36	38	40
10	.05	4	7	11	14	17	20	24	27	31	34	37	41	44	48	51	55	58	62
	.01	1	3	6	8	11	13	16	19	22	24	27	30	33	36	38	41	44	47
11	.05	5	8	12	16	19	23	27	31	34	38	42	46	50	54	57	61	65	69
	.01	1	4	7	9	12	15	18	22	25	28	31	34	37	41	44	47	50	53
12	.05	5	9	13	17	21	26	30	34	38	42	47	51	55	60	64	68	72	77
	.01	2	5	8	11	14	17	21	24	28	31	35	38	42	46	49	53	56	60
13	.05	6	10	15	19	24	28	33	37	42	47	51	56	61	65	70	75	80	84
	.01	2	5	9	12	16	20	23	27	31	35	39	43	47	51	55	59	63	67
14	.05	7	11	16	21	26	31	36	41	46	51	56	61	66	71	77	82	87	92
	.01	2	6	10	13	17	22	26	30	34	38	43	47	51	56	60	65	69	73
15	.05	7	12	18	23	28	33	39	44	50	55	61	66	72	77	83	88	94	100
	.01	3	7	11	15	19	24	28	33	37	42	47	51	56	61	66	70	75	80
16	.05	8	14	19	25	30	36	42	48	54	60	65	71	77	83	89	95	101	107
	.01	3	7	12	16	21	26	31	36	41	46	51	56	61	66	71	76	82	87
17	.05	9	15	20	26	33	39	45	51	57	64	70	77	83	89	96	102	109	115
	.01	4	8	13	18	23	28	33	38	44	49	55	60	66	71	77	82	88	93
18	.05	9	16	22	28	35	41	48	55	61	68	75	82	88	95	102	109	116	123
	.01	4	9	14	19	24	30	36	41	47	53	59	65	70	76	82	88	94	100
19	.05	10	17	23	30	37	44	51	58	65	72	80	87	94	101	109	116	123	130
	.01	4	9	15	20	26	32	38	44	50	56	63	69	75	82	88	94	101	107
20	.05	11	18	25	32	39	47	54	62	69	77	84	92	100	107	115	123	130	138
	.01	5	10	16	22	28	34	40	47	53	60	67	73	80	87	93	100	107	114

The values refer to the probability of $\alpha \leq 0.05/0.01$ (n_1= group with a lower number of cases; n_2= group with a greater number of cases).

2.5.3 Spearman's rank correlation coefficient

Spearman's rank correlation coefficient is used to identify and test the strength of a relationship between two sets of data.
The Spearman correlation coefficient, ρ (rho), can be calculated when the two variables (X; Y) are in terms of ranks. The null hypothesis is that ρ is 0. A ρ= 0 means that the ranks of one variable do not co-vary

with the ranks of the other variable; in other words, as the ranks of one variable increase, the ranks of the other variable do not increase (or decrease).

If the change in one variable brings about a change in the other variable, they are said to be correlated. A $\rho = +1$ means that the two variables are directly correlated (low values of X correspond to low values of Y); A $\rho = -1$ means that the two variables are indirectly correlated (high values of X correspond to low values of Y) (Soliani, 2001).

3. Results

3.1 Analyses of PAR-PARP and membrane lipid profiles

3.1.1 PAR-PARP

Automodification of PARP (PAR-PARP) was evidenced by immunoblotting with polyclonal anti-PARP catalytic site antibodies (PARP-*CS*Abs). These Abs allowed to visualize the enzyme in both native and modified form. Figure 7 shows the patterns of unmodified PARP from three analysed samples (Figure 7A, a, lanes 1-3). In the absence of automodification the immunoband corresponded to native PARP. In Figure 7A (b, lanes 1-3) three examples of automodified PARP (PAR-PARP) from other samples are shown. PAR-PARP gave signals above 113 kDa. The shift of molecular mass accounted for more than hundred ADP-ribose molecules modifying the enzyme.

A worth noting result was that in lymphocytes the PARP enzyme involved in automodification was mainly PARP2 (62 kDa; Figure 7A, a).

Confirming this finding was an experiment where PAR-PARP was excised from the gel and the slice incubated under alkali conditions. The ester bond between protein and the first unit of poly(ADP-ribose) is highly sensitive to alkali treatment. The polymer-free PARP was eluted, electrophoresed and re-analysed by the same antibodies (Figure 7B); it gave a band shifted from high molecular weight (above 113 kDa), to 62 kDa, the molecular mass of PARP2 (Figure 7B, 1'-3').

Following this experimental procedure, the lymphocyte PARP was analysed for all subjects and the amount of automodification was measured by densitometry of the bands, expressed as optical density/mm^2 (Figure 7C). DNA damage levels of the samples with high PAR-PARP were measured by comet assay (data not shown). The results confirmed that PARP automodification increased as consequence of DNA breaks.

Being the level of PARP automodification a measure of DNA damage, the results of densitometry allowed to classify subjects according to the extent of DNA damage.

Controls were identified from anamnestic data, whose levels of PAR-PARP allowed to define the limits of variability of the reference

range. The pathological samples were analyzed and compared with the controls. The density of immunopositive bands of pathological samples were higher than the controls, and differed from each other, indicating different levels of DNA damage.

The lymphocyte PARP was analyzed for all subjects and the results were grouped according to automodification levels: OD<20000 int/mm^2 corresponded to physiological PAR-PARP (PA); 20000≤OD≤40000 int/mm^2 indicated moderate levels of PAR-PARP (MA); hypermodification of PAR-PARP (HA) gave OD>40000 int/mm^2.

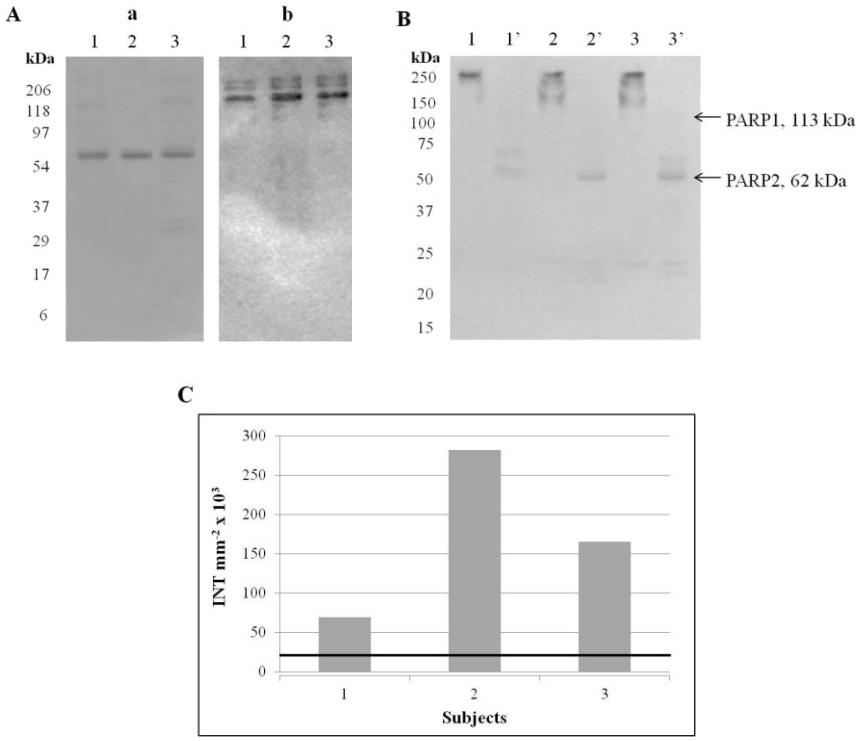

Figure 7: **Anti-PARP catalytic site immunoblotting of lymphocyte lysates.**
Panel A, anti-PARP immunoblottings of three samples with native (a) and automodified (b) PARP. Panel B. Immunopatterns in A, (b), before (1, 2, 3) and after (1', 2', 3') alkali incubation of PAR-PARP. In 1', 2', 3' poly(ADP-ribose)-free PARP localizes at the molecular mass of native enzyme. Panel C. Densitometric analysis of immunoblots of control samples (black) and pathologic (gray) of the panel A(a) and A(b), respectively.

3.1.2 Membrane fatty acid analysis

The erythrocyte membrane has a characteristic FA asset, i.e., saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA), *cis*/*trans* isomers, all present in specific amounts in the phospholipids of the bilayer.

The response of lipid analysis is the FAT PROFILE, a table with the fatty acid values found in the erythrocyte membranes in comparison with the normal values (Table 2). The values are expressed as a relative percentage (% rel) of the total fatty acids considered.

Table 2: **Main fatty acids and optimal intervals determined in the erythrocyte membrane of healthy subjects.**

Fatty Acids	Normal ranges (% rel.)
Palmitic (16:0)	17 - 27
Palmitoleic (16:1)	0.2 - 0.5
Stearic (18:0)	13 - 20
Oleic (18:1)	9 - 18
Trans 18:1	0 - 0.3
Vaccenic (18:1)	0.7 - 1.3
Linoleic (18:2) (omega 6)	9 - 16
Ecosatrienoic (20-3) (omega 6)	1.9 - 2.4
Arachidonic (20:4) (omega 6)	13 - 17
Mono Trans ARA	0 - 0.4
EPA (20:5) (omega 3)	0.5 - 0.9
DHA (22:6) (omega 3)	5 - 7
Tot. saturated (SFA)	30 - 45
Tot. monounsaturated (MUFA)	13 - 23
Tot. polyunsaturated (PUFA)	28 - 39
SFA/MUFA ratio	1.7 - 2
Omega 6/omega 3 ratio	3.5 - 5.5

Table 2 includes the main SFA (Palmitic and Stearic acids), MUFA (Palmitoleic and Vaccenic acids) and the main PUFAs. From these values it is possible to calculate the SFA/MUFA and ω6/ω3 ratios. The ratio SFA/MUFA can indicate biosynthetic and dietary contribution, which create the balance between these two FA families in the membrane compartment, regulating structural organization, biophysical properties and functioning. The harmful consequences of

an excess of SFA for health is known, as well as favorable membrane biophysical properties of fluidity and permeability, and biochemical functions and signaling cascades, depends on the natural double bond content (MUFA, PUFA). It is worth underlining that the unsaturated content is correlated to the functioning of enzymes, namely Δ5-, Δ6- and Δ9-desaturases involved in PUFA metabolism. Fat profile is a tool to check either FA enzyme metabolic pathways and the pro- and anti-inflammatory state of the cell. Moreover this analysis is able to measure the levels of *trans*-PUFA, a direct index of oxidative stress.
The profile is completed with a graphic to connect the measured values with anamnesis and metabolism of the patient (Figure 8A), and with a Membrane Unbalance Index (MUI) that, by an algorithm visualized by a color scale, summarizes simply the FA unbalance percent (Figure 8B). For the Italian population, the reference values of erythrocyte membrane fatty acids have been described and are reported in Table 2.

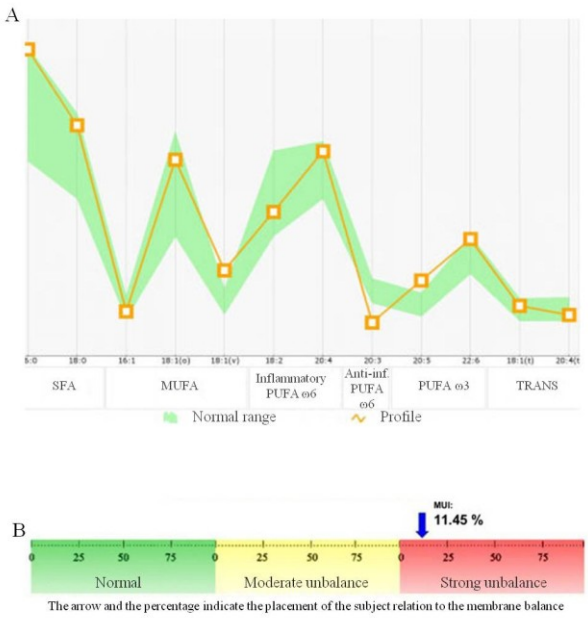

Figure 8: **A. Fatty acid profile of a subject (orange line) compared to normal ranges (green zone). B. Membrane Unbalance Index (MUI) and colored scale.**

3.2 Relationship of PARP automodification and membrane lipid composition with lifestyle and health status

The study performed on 84 subjects undergoing endoscopy, by detecting PAR-PARP levels and analyzing erythrocyte membrane fat profiles, started monitoring the physio-pathological state of the cell, and its possible correlation with lifestyle, diet or diseases.

In the following paragraphs the results of PAR-PARP levels and fat profiles are reported and statistically evaluated separately.

3.2.1 Automodification of PARP

The number of analysed subjects changed over time. The lymphocyte PARP was initially analyzed for 70 subjects; thereafter this number increased to 84. The results were grouped according to automodification levels: OD<20000 int/mm^2 corresponded to physiological PAR-PARP (PA); 20000≤OD≤40000 int/mm^2 indicated moderate levels of PAR-PARP (MA); hyper-modification of PAR-PARP (HA) gave OD>40000 int/mm^2.

The results of analysis of variance were comparable for both the 70 subjects and the same group increased to 84 samples (Figure 9B).

By this analysis the difference between PA, MA and HA was statistically significant (Figure 9A).

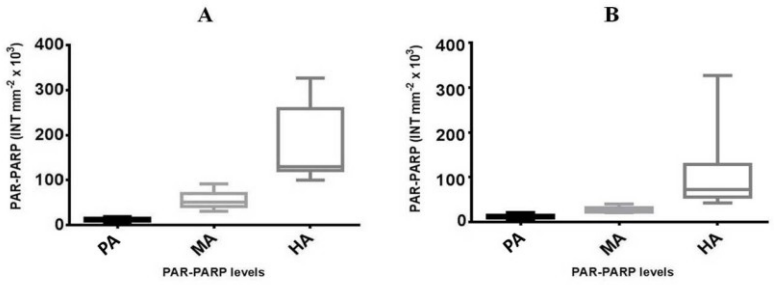

Figure 9: **Analysis of variance of PAR-PARP levels.**
A. PA, Physiological PAR-PARP (*n*=24); MA, Moderate PAR-PARP (*n*=21); HA, High PAR-PARP (*n*=25). **B.** PA (*n*=28); MA (*n*=22); HA (*n*=34).
A high statistical significance of the difference between the three groups was calculated, with P<0.0001.

The three groups had values significantly different each other; the PAR-PARP level range was narrow in PA and MA groups, whereas those of HA group were distributed in a wider range. These ranges were reduced by increasing the number of subjects from 70 to 84 (Figure 9B). However, the difference between the three groups was still statistically significant.

Increased PAR-PARP levels were measured in case of inflammation, viral infections and cancers with a relevant clinical activity (no surgical or therapeutic treatment, presence of metastases). As opposite PA was measured in healthy and remitting patients.

On this basis the first analytical approach was to group the data by BMI. Three groups were identified: normal weight (19<BMI<25; NW); overweight (BMI<30; OW); obese (BMI>30; O).

The comparison of PAR-PARP levels with BMI groups (NW, OW, O) gave, either within each group or between the three groups, *P* values highly above 0.1, i.e. the difference was not statistically relevant (Figure 10). χ^2 test confirmed this behavior (Table 3). Similar results were obtained, comparing PAR-PARP levels and the age of volunteers (data not shown).

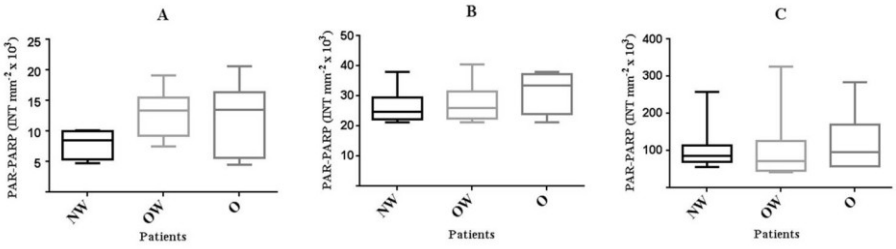

Figure 10: **PAR-PARP levels in BMI groups.**
NW=normal weight (*n*=22); OW=overweight (*n*=41); O=obese (*n*=21). The difference between groups was not significant (P>0.05).

Table 3: χ^2 **non parametric test of PAR-PARP compared to BMI of subjects.**

	NW	OW	O
χ^2	1.37	3.23	2.05
n	22	41	21
P	1	1	0.999

NW. Normal weight; OW. Over weight; O. Obese.
n= number of cases.

In conclusion, even before performing other more complex diagnostic analyses, and in accordance with data from literature, DNA damage is easily detectable by PAR-PARP measures and is independent from anthropometric data.

However, by grouping the examined subjects in three ranges of age (years 20-39; 40-59; >60) and considering anamnestic data, the results of Table 4 were obtained.

Table 4: **Age-related PAR-PARP levels.**

	MA			HA		
Age (Years)	20-39 (%)	40-59 (%)	> 60 (%)	20-39 (%)	40-59 (%)	> 60 (%)
No Pathology	25 (Smokers)			10 (Obese)		
Allergy	50	40	20	27		
Hypertension/Dyslipidemias	25	60	80			
Fibromatosis/Polyposis				27	50	50
Thyroid disease/Autoimmunity				27	30	15
Viral infections				9 (HPV)	20 (HCV)	20 (HCV)
Cancer						15

Percentage (%) refers to the number of affected subjects per each group of PAR-PARP levels.
HPV: Human Papilloma Virus; HCV: Hepatitis C virus.

The youngest people (years 20-39) showed mainly moderate PAR-PARP, at the highest percentage (50%) for allergy, and 25% for either smoking or dyslipidemia/hypertension. The latter increased to 60% and 80% for the other two ageing groups (40-59, >60, respectively). High PAR-PARP (HA) had noticeable incidence at any age for adenomas, autoimmunity and HCV/Papilloma virus infections. It occurred in 15% of aged (years >60) patients with cancer.

Physiological levels of PAR-PARP were measured either in the absence of diseases, or in cases of diseases under remission or therapeutic treatments. It is worth noting that both moderate and high PAR-PARP levels were observed mostly in subjects with inflammatory conditions of lower or higher gravity (Table 4), but all relatable to different DNA damage extents.

3.2.2 Membrane lipid profile (fat profile)

The fatty acids taken from fat profiles as indexes of cell functions and metabolism included the main SFA and MUFA, and omega 3 (EPA, DHA), omega 6 (DGLA, AA). In order to evidence specific features of FA metabolic enzymatic pathways, the ratio of SFA/MUFA, and of ω6/ω3 PUFA were considered. In particular, on the basis of normal values indicated by fat profile, three groups of subjects were identified: physiological, with FA within the normal range; < min, for those subjects with values below the lowest range limit; > max, if the values were above the highest range limit (Table 5, A-C). A further subdivision was made by considering the three BMI groups (NW, OW, O), Table 5 (A-C). In this way for each BMI group it was possible to highlight both the different unbalance level of fatty acids and the number of patients with altered FA amounts.

Table 5: **Different levels of the main fatty acids in NW (A), OW (B) and O (C) groups.**

A. Normal weight (*n*= 22)

Patients	Physiological values						< min value					> max value					
(*n*)	S/M	ω6/ω3	EPA	DHA	DGLA	AA	S/M	ω6/ω3	EPA	DHA	DGLA	AA	S/M	ω6/ω3	EPA	DHA	DGLA	AA
6				5.3	2.2				0.3				2.4	5.55				19.3
12			0.9			14.2				4.3	1.6		3.2	5.67				
13		4.71		5.1	2.4	13.3			0.4				2.8					
15						13.3				2.4			2.4	7.11	1.2		2.8	
17			0.5			14.7				2.6	1.6		2.6	8.10				
20	1.9					14.8				4	1.6			6.20	1			
25			0.7			16.3				3.3			2.2	8.25			3.7	
26	2		0.8		2.4	16.3				4.4				5.98				
35	2	4.77	0.6	5.1	2.1	16.4												
39			0.8		2.2	16.6				3.5			2.1	6.47				
44				6.7	2.3	14.3		3.13					2.5		1.6			
57			0.5		2.1	15.4				3.8			2.6	7.78				
65		4.77	0.9	5.1		14.9					1.7		2.5					
74		3.85	0.8		2.3								2.5			7.1		18.1
76		4.09	0.9	6.5									2.6				2.7	18.1
78		5.13	0.9		1.9	16.8				4.7			2.5					
79					1.9	13.1			0.4	3.9			2.4	7.07				
90						15.8			0.4	3.4	1.3		2.6	8.10				
91						15.9				4	1.7		2.5	7.79	0.2			
I			0.8			14.2				2.8	1.5		2.3	7.53				
IV						15.7				4.2	1.8		2.5	5.62	1			
VII		4.38	0.6	5.2		13.2							2.2				2.5	

Normal ranges of FA (%): SFA/MUFA (1.7-2); ω6/ω3 (3.5-5.5); EPA (0.5-0.9); DHA (5-7); DGLA (1.9-2.4); AA (13-17).

B. Overweight (*n*= 41)

Patients	Physiological values						< min value						> max value					
(*n*)	S/M	ω6/ω3	EPA	DHA	DGLA	AA	S/M	ω6/ω3	EPA	DHA	DGLA	AA	S/M	ω6/ω3	EPA	DHA	DGLA	AA
3		5.16	0.5	6.3									3.1				3.1	20.8
7		5.00	0.9	5.5	2								2.5					20.3
8		5.05	0.6	5.4	2								2.6					19.9
9		3.71	0.9	6.8							1.8		2.3					19
11		4.01		6.7	2	16.6			0.4				2.9					
16			0.6		1.9	14.7							2.3	6.12		7.7		
24	2	4.90		5.1											1.1		2.6	17.9
28	2	5.00	0.9	5	2.4	14.8												
30	1.9	4.98	0.7	5.5		17					1.6							
32	2			6.6	2.3	15.8	3.35								1.8			
36			0.5			16.7	3.41				1.6		2.3			7.8		
37		4.14	0.8	5.6		15.7					1.6		2.6					
38			0.6		2					4.1			2.5	6.23				17.1
40			0.9		1.9	15.3				3.5			2.2	6.32				
41	2	5.15			1.9	17				4.8					1.6			
45		4.41		5.5		15.9					1.7		2.7		1.1			
46		4.97	0.9	5	2.3								2.3					17.1
47				5					0.4		1.4		2.2	6.24				19.7
48		4.16		5.9		17							2.5		1.4		2.5	
49		5.45	0.5	5.1							1.1		2.3					17.9
50		3.99		5.8	2.2	16							2.1		1.3			
53		3.96	0.8	6.4		16					1.5		2.6					
55		4.15	0.6	6.1	2.4	14.7							2.9					
58					2.2				0.4	3.6			2.2	7.58				17.6
62			0.5			15.7				4.5			2.3	5.94			3.2	
63		3.74		6.2	2.3	15.5							2.6		1.6			
64		5.06	0.7	5.5		17							2.9				3	
66		4.44	0.9	6.4	2.2	15.5							2.8					
67					2.3	16.4	2.97						2.5		1.4	8.8		
68		3.97	0.9	6.2		16.5					1.7		2.2					
69			0.5							4.7			2.3	6.12			2.7	17.6
71						13.7			0.4	3.8			2.5	6.21			2.5	
72			0.5		2.2	13.3				2.7			2.1	8.84				
75			0.9							4.4			2.7	5.74			2.6	17.2
77		5.38		5							1.6		2.9		1			19.1
80		3.63		6.4	2.4	16.3							2.7		1.5			
82		5.21	0.9			16.4				4.8	1.5		2.8					
83		4.31			2	13.8				4.9			2.5		1			
86			0.7		2.2	15.5				2.5			2.5	9.38				
V		4.34	0.8	5.5	2	15.3							2.8					
VI		4.95	0.7			15.2				3.9	1.5		2.6					

C. Obese (*n*= 21)

Patients	Physiological values						< min value						> max value					
(*n*)	S/M	ω6/ω3	EPA	DHA	DGLA	AA	S/M	ω6/ω3	EPA	DHA	DGLA	AA	S/M	ω6/ω3	EPA	DHA	DGLA	AA
5			0.5			16.9				4			2.8	6.98			3.2	
21		5.42	0.5		1.9					4.8			2.1					18.5
22						14.5				3.8			2.2	5.83	1.4		3.3	
23	2				2.3	16				3.5				6.44	1.3			
27		4.68		5.6							1.3		2.5		1			18.1
29			0.8		2.3	15.5				3.5			2.3	7.16				
31				6.9		13.1		3.25			1.6		2.3		1.1			
52		4.92			2.2	14.7				4.9			2.2		1.1			
54			0.5			16.4				4.6			2.5	6.02			3	
60						13.2		3.16			1.5		2.8		1.5	7.1		
61		4.33		5.3		16.8					1.7		2.3		1			
73		4.06		6	1.9								2.6		1			17.8
81			0.5		1.9					4.2			2.3	6.55				19.7
84		3.67				13.2				4.3	1.3		3.1		2.7			
85			0.7			16.9				4.3	1.3		2.5	5.96				
87			0.6		2.2	16.1				3.1			2.5	7.78				
88			0.6		2.1	15.5				3.4			2.5	7.60				
89		4.90	0.9	5.1	2	15.4							2.4					
92			0.7		1.9					3.9			2.3	7.11				18.2
93										4	1.7		2.5	9.00	0.2			17.1
II				6.7		13.8		3.29					2.7		1.6		3	

SFA/MUFA ratio. The unbalance of SFA/MUFA ratio occurred for all subjects grouped for BMI. An increase of SFA was recorded for almost all the normal weight subjects (86% of cases); the values were within the normal range for 14% people; no patients had SFA levels lower than normal values.

In overweight subjects 88% SFA/MUFA ratios were altered, whereas 12% ones were physiological.

The same trend was recorded in the obese group. Except for one subject, the other ones showed an increase of SFA/MUFA ratio.

ω6/ω3 ratio. Excessive values of ω6 were found in more than half of the normal weight people; physiological measures were found in 32% of case; only one subject had a lower ω6/ω3 ratio. The increase in the ratio was mainly due to low levels of DHA in 14 of 22 people. The levels of EPA and AA fell within in the normal ranges for almost all subjects, (59%, and 82% of cases, respectively). Physiological DGLA values were found for only 45% of cases.

Physiological levels of ω6/ω3 ratio were measured for 86% of OW subjects, they were due to the normal values of EPA, DHA, and DGLA found in over half of the group (for 26, 25 and 21 of 41 people, respectively).

The slight increase of the ω6/ω3 ratio for 52% of the obese subjects

was mainly due to a decrease of DHA values for 67% of them.
AA was within normal range in almost all the subjects of the three groups.
The unbalance of the ratios of SFA/MUFA and ω6/ω3 PUFA were highlighted in graph (Figure 11, A-C). The values were compared to the normal ranges, drawn by the horizontal lines to the x axis. In any BMI group there was a plain unbalance of SFA/MUFA ratios compared to the control values; even the ω6/ω3 ratio was altered in the majority of subjects recruited, regardless of BMI. χ^2 test confirmed these results (Table 6).
These results at least allow correcting the quality of food, limiting the intake of nutritional sources of SFA, in order to re-establish the right content of these FA categories. Thereafter, it is possible to evaluate whether there might be alterations in any enzymatic step of FA metabolism requiring further attention.
A high unbalance was measured for ω6/ω3 PUFA. In some cases, there was a net increase of arachidonic acid, a precursor of many pro-inflammatory compounds, compared to the expected normal ranges.
Mostly a high reduction of ω3 PUFA was observed. Due the anti-inflammatory value of these FA, their deficiency indicated a low anti-oxidant defense ability of cells, and a high risk of inflammation.

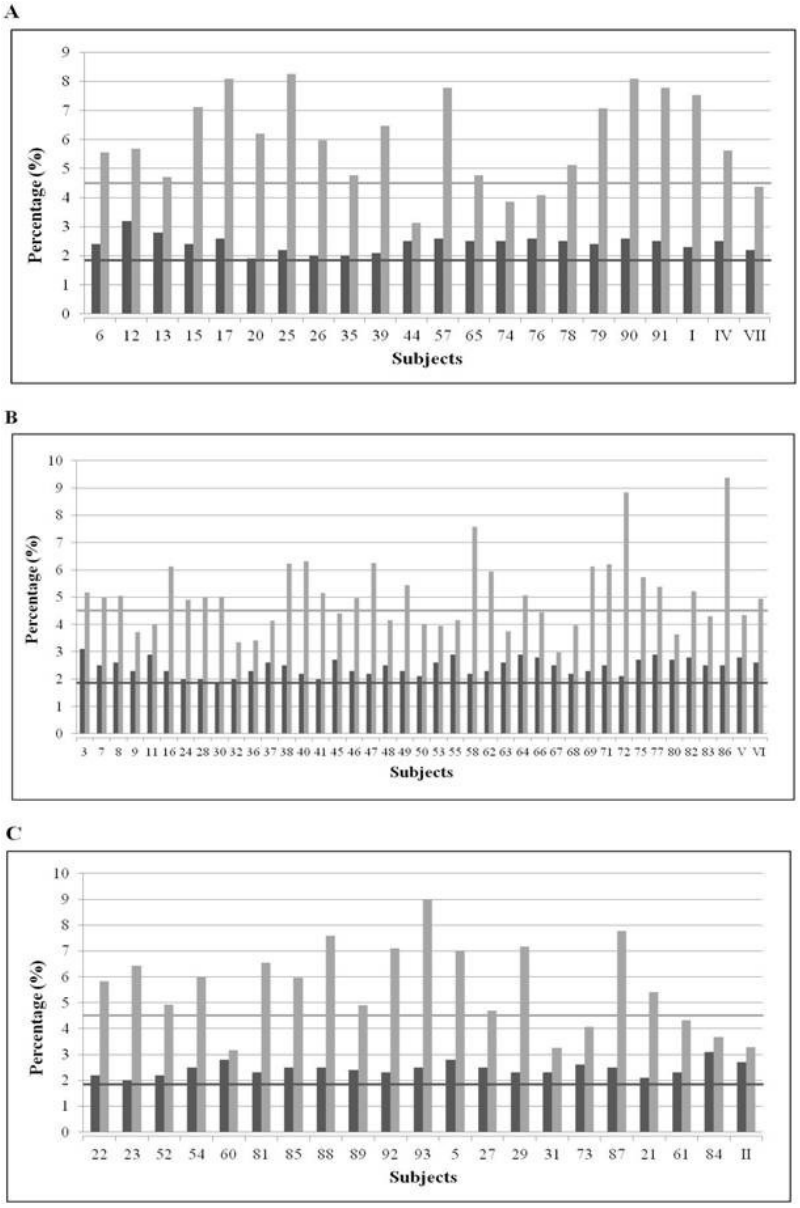

Figure 11: **SFA/MUFA and ω6/ω3 PUFA unbalance in the recruited volunteers.**

The values measured for each patient versus normal values for the three BMI groups: **A.** Normal Weight; **B.** Over Weight; **C.** Obeses.

SFA/MUFA ■ ω6/ω3 ■

Cut off of normal ranges SFA/MUFA ▬ and ω6/ω3 ▬

Table 6: χ^2 **non parametric test of SFA/MUFA ratio compared to BMI of subjects.**

	NW	OW	O
χ^2 (S/M)	4.85	10.13	4.77
n	22	41	21
P	0.999	1	0.991

NW. Normal Weight; OW. Overweight; O. Obese.
n= number of cases.

3.2.3 Correlation between PAR-PARP results and fat profiles in BMI groups

In order to answer one of the question stated by the aim of this work, the results obtained by the two analyses were statistically evaluated to check whether they could correlate each other, and be used in combination to detect the physio-pathological state of the cell. The Spearman test was performed, giving the results shown in Table 7. Taking into account that the Spearman's rank correlation coefficient (ρ) is the main parameter, and that values of ρ equal to 1 indicate a direct correlation, whereas values of -1 show an indirect correlation, the test did not reveal a significant correlation in any of three groups (Table 7).

The results described in section 3.2.1 indicated that PAR-PARP levels allow to detect DNA damage occurrence, that is independent from anthropometric data.

Table 7: Correlation between PAR-PARP levels and fat profile in BMI groups.

A. Normal weight

	PAR-PARP vs. DHA	PAR-PARP vs. EPA	PAR-PARP vs. DGLA	PAR-PARP vs. S/M	PAR-PARP vs. AA	PAR-PARP vs. ω6/ω3
ρ	0.151	0.012	0.342	-0.270	-0.039	-0.099
P	0.502	0.959	0.120	0.224	0.863	0.662
Significance (alpha = 0.05)	No	No	No	No	No	No
Number of pairs	22	22	22	22	22	22

B. Overweight

	PAR-PARP vs. DHA	PAR-PARP vs. EPA	PAR-PARP vs. DGLA	PAR-PARP vs. S/M	PAR-PARP vs. AA	PAR-PARP vs. ω6/ω3
ρ	-0.162	-0.059	-0.182	-0.305	0.236	0.124
P	0.312	0.716	0.256	0.053	0.138	0.439
Significance (alpha = 0.05)	No	No	No	No	No	No
Number of pairs	41	41	41	41	41	41

C. Obese

	PAR-PARP vs. DHA	PAR-PARP vs. EPA	PAR-PARP vs. DGLA	PAR-PARP vs. S/M	PAR-PARP vs. AA	PAR-PARP vs. ω6/ω3
ρ	0.161	0.194	0.089	0.193	-0.035	-0.214
P	0.486	0.399	0.702	0.402	0.879	0.351
Significance (alpha = 0.05)	No	No	No	No	No	No
Number of pairs	21	21	21	21	21	21

3.3 PARP automodification and membrane lipid composition in patients with inflammatory pathologies

The rationale of the second approach used to evaluate the results of both analyses obtained for 95 subjects was to consider them in relationship with clinical data, i.e. upon possible occurrence of any pathology. Therefore, the data were treated independently from BMI, taking into account the declared/diagnosed diseases.

This phase of the work was developed following the steps of the previous chapter (3.2). First, non parametric tests were performed for results of PAR-PARP measures by comparing the altered values with the physiological ones.

Second, fat profile results were statistically evaluated by comparing unbalanced levels of FA with the normal ones. In this second phase of the work the attention was focused mainly on PUFAs (EPA, DHA, DGLA, AA), considered as single FAs, rather than only on the ratio of SFA/MUFA and ω6/ω3.

Third non parametric tests were performed to study the possible correlation of the two combined analyses, PAR-PARP and fat profile, towards a given pathology.

The results of the molecular analyses (PAR-PARP and fat profile) were compared with anamnestic and clinical data of the volunteers (84 subjects of the previous analyses, reported in section 3.2, plus 11 new patients).

All these subjects were different as some ones had no pathology, few ones had a single pathology, and a larger number declared more than one disease. It was necessary to enclose them in different groups and subgroups as detailed below. In this way groups and subgroups were often composed of few subjects. For this reason, non parametric tests had to be used in order to perform rightly statistical analyses. They gave the results described in the following sections.

3.3.1 Groups of enrolled subjects

In Table 8 general information of the 95 subjects is highlighted.

Table 8: Distribution of recruited subjects (*n*=95) by gender, age and some lifestyle habits.

Gender	Male	Female
Number (%)	57 (60%)	38 (40%)
Age (20-39)	7 (12%)	9 (24%)
Age (40-60)	24 (42%)	13 (34%)
Age (> 60)	26 (46%)	16 (42%)
Smokers	14 (25%)	9 (24%)
Physical Activity (Soft)	21 (37%)	13 (34%)
Physical Activity (Moderate)	18 (32%)	12 (32%)
Physical Activity (Hard)	10 (18%)	---

6 subjects out of 95 had no pathology as stated in their interviews and confirmed by clinical analyses; by comparison of these data with PAR-PARP and fat profile, they were considered as controls (healthy).

The subdivision of patients in different groups was first made on the basis of the diagnosed/declared disease, if unique, or the most serious one occurring and then, within the same pathology, subgroups were identified, starting from patients with a single disease. In those cases where pathologies of a different type were co-present, a separate subgroup named "mixed" was considered.

The subjects were grouped as reported in Table 9. Group 0 corresponded to volunteers without diseases as a result of their statement and from clinical and diagnostic analyses. This group was taken as healthy.

The most numerous group enclosed patients with gastro-enteric diseases (group 1, *n*=37). Subjects in the subgroup 1a had diagnosis of simple or hyperplasic polyposis; patients in the subgroup 1b showed adenomas and hyperplastic polyps; the subgroup 1c consisted of patients affected by non-cancerous gastrointestinal disorders; patients in the subgroup 1d were affected by gastro-enteric cancer; those of the subgroup 1e had other disorders along with the cancer and were defined "mixed".

Group 2 was composed of patients with mild thyroid disorders (2a), subjects with the autoimmune Hashimoto's thyroiditis (2b) and the "mixed" ones with thyroid disorders and other diseases (2c). The groups of patients affected by allergies and HCV were divided into two subgroups including the "mixed".
The remaining subjects were grouped as affected by autoimmunity (group 5), no gastro-enteric cancer (group 6), cardiopathy (group 7), dyslipidemia/hypertension (group 8). Details of diseases in each group are given in the footnote to Table 9.

Table 9: Groups of patients.

Group	*n*	Pathology	Subgroup	Disease	*n*
0	6	Healthy (controls)	-----	-------	6
1	37	Gastro-enteric (GE)	1a	Polyposis / hyperplasic polyposis	9
			1b	Hyperplasic polyposis with adenomas	7
			1c	Other GE diseases	8
			1d	GE cancer	7
			1e	Mixed GE cancer	6
2	10	Thyroid	2a	Nodules, goiter, hyper-, hypo-thyroidism	4
			2b	Autoimmune Hashimoto's thyroiditis	3
			2c	Mixed	3
3	7	Allergy	3a	Asthma, pollen	4
			3b	Mixed	3
4	7	HCV	4a	HCV	3
			4b	Mixed	4
5	4	Autoimmunity	---	Psoriasis, anti-P-lipid Ab syndrome, rheumatoid arthritis	4
6	12	Other cancers (no GE)	---	Uterus, prostate, ear	12
7	3	Cardiopathy	---	Stenosis, ischemia, vascular p.	3
8	9	Dyslipidemia/Hypertens.	----	----	9

(1e): cancer GE, thyroid disorders, allergy, hypertension, dyslipidemia;
(2c): thyroid disorders, allergy, hypertension, dyslipidemia, polyposis;
(3b): allergy, hypertension, uterine fibroid, reflux;
(4b): HCV, thyroid disorders, polyposis, hypertension, adenoma, cancer, cardiopathy, dyslipidemia.

3.3.2 Statistical analysis by Receiver Operating Characteristic (ROC) curve

3.3.2.1 ROC curves of controls

The values of PAR-PARP and EPA, DHA, DGLA, AA of the control subjects (group 0) were compared to the levels of physiological PAR-PARP and normal PUFAs known from literature. Figure 12 shows their ROC curves. The areas below the curves of controls (healthy) had values equal or close to AUC 0.5, meaning that they could be considered as physiological. Indeed they were taken as reference to evaluate the statistical significance of the data obtained for the other groups.

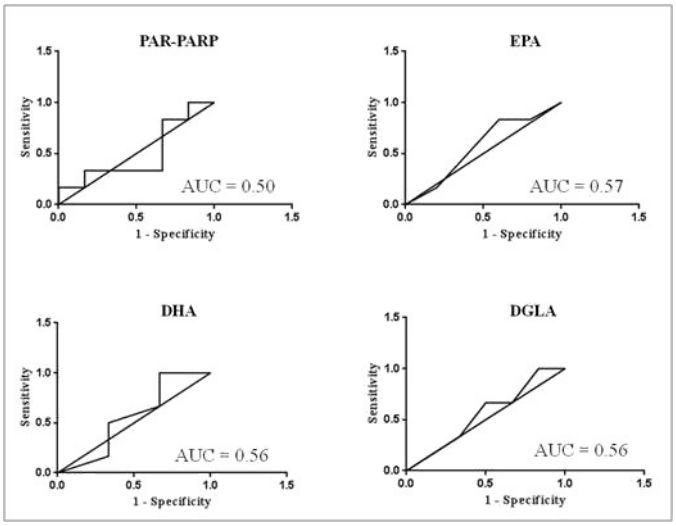

Figure 12: **ROC curves of PAR-PARP levels and PUFAs to define control subjects.**

The area of PAR-PARP was exactly 0.5, whereas PUFAs gave a maximum of 0.57, taken as an index of unbalance in non pathological conditions possibly related to lifestyle.
These results indicated that the six subjects identified as controls had PAR-PARP and PUFA levels within or slightly out the physiological values reported in literature.

3.3.2.2 Analysis of PAR-PARP data by ROC curves

The results of automodified PARP from each group, compared to group 0 (controls), were statistically evaluated by drawing the corresponding ROC curve (Figure 13).

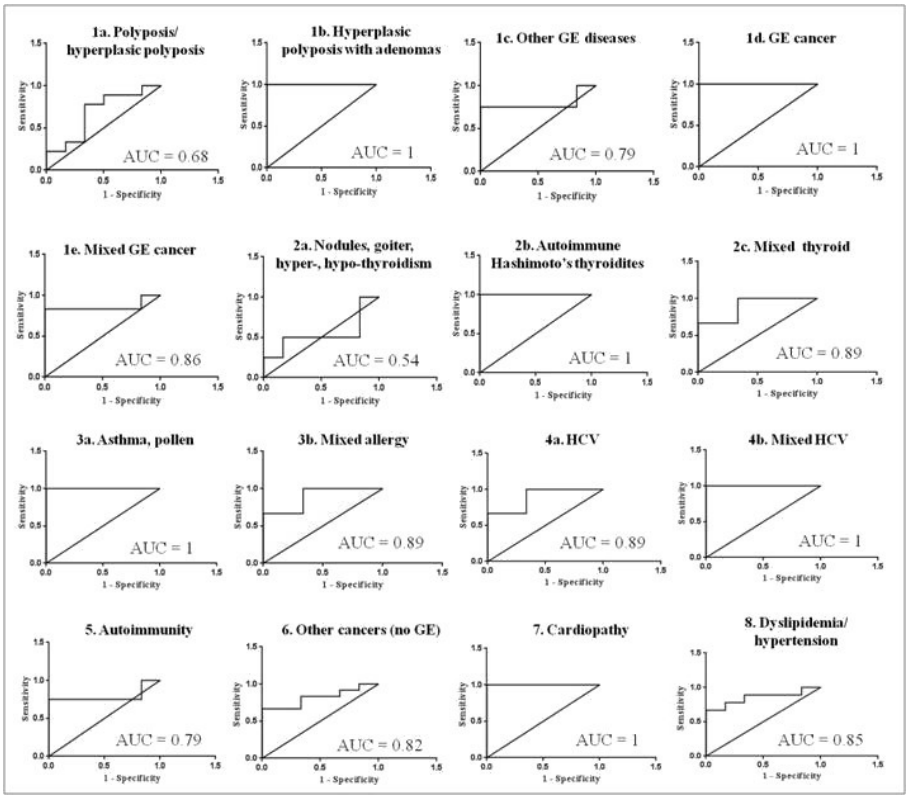

Figure 13: **ROC curves of PAR-PARP levels in subjects with different pathologies.**

In most of the groups and subgroups the ROC curve areas of PAR-PARP were higher than 0.7. Except for the subgroups of subjects with polyposis (1a) and mild thyroid diseases (2a). A very slight significance was evidenced for infections, thyroid diseases, cancers under treatment and GE carcinomas. Areas under ROC curves highly increased mainly in HCV and GE adenomas. Intermediate values were observed for the other groups.

3.3.2.3 Analysis of PUFAs by ROC curves

The ROC curve was determined for EPA, DHA, as ω3, and DGLA, which is the ω6 FA at the cross-road between pro-inflammatory (AA biosynthesis) and anti-inflammatory (PGE_2 biosynthesis) pathways.
Figures 14, 15 and 16 show, respectively, the ROC curve areas of EPA, DHA, and DGLA, for the different groups compared to their controls.

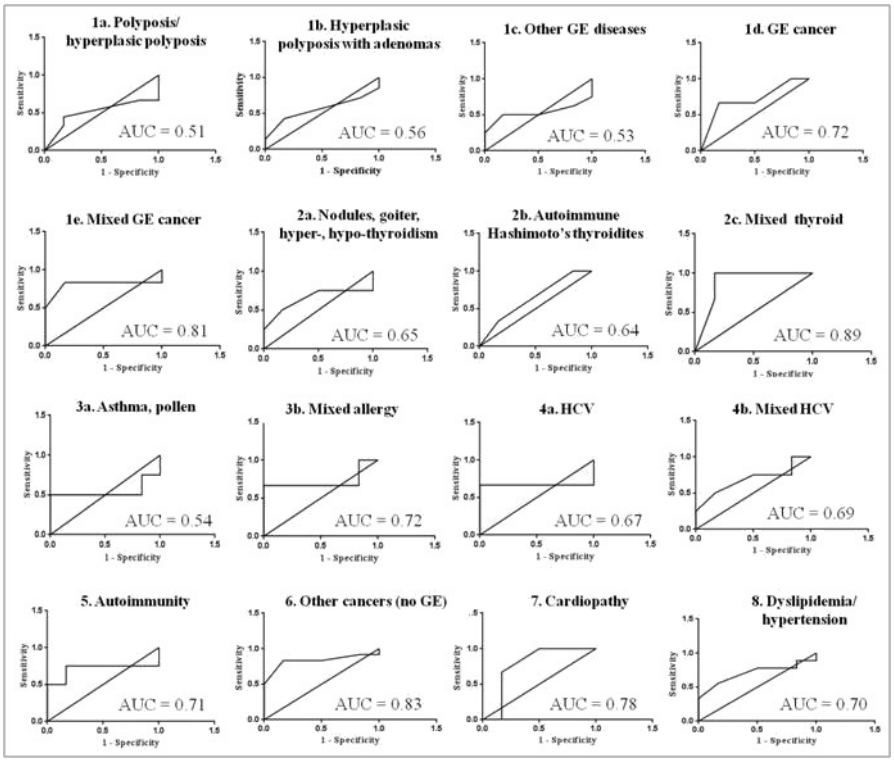

Figure 14: **ROC curves of EPA values in patients with different pathologies.**

The greatest discriminating power of the test had occurred for the groups with cancer, cardiopathies and autoimmunity. A very high AUC value was found also for the patients in group 2c ("mixed" thyroid), probably due to interference of the other ongoing pathologies, especially dyslipidemia and hypertension.

The ROC curve areas (AUC) were not particularly significant for the other groups.

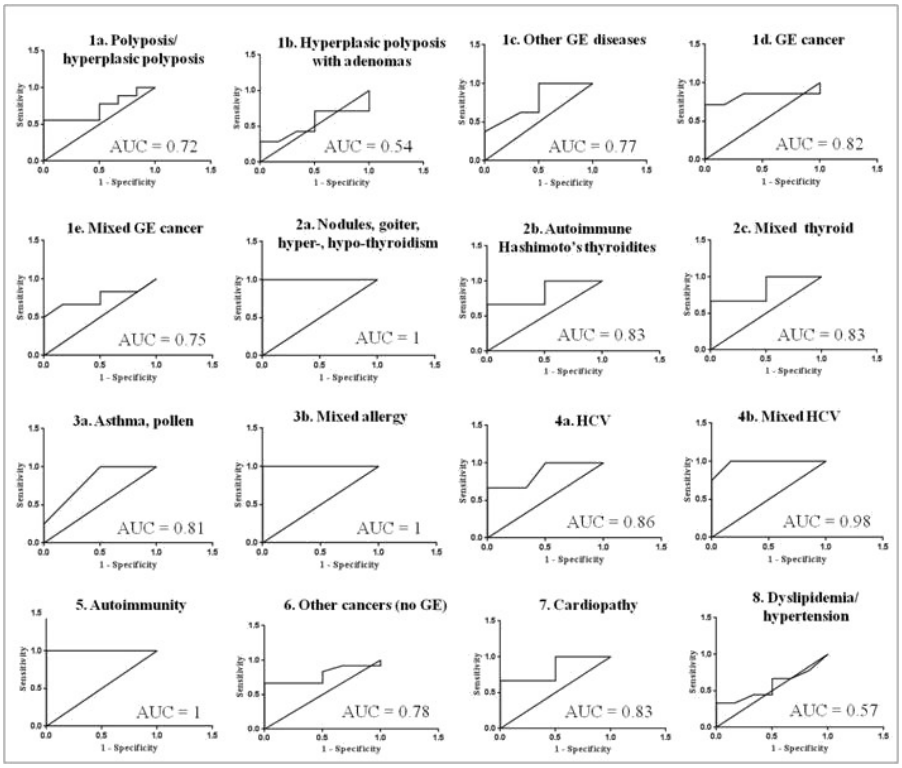

Figure 15: **ROC curves of DHA values in patients with different pathologies.**

In most groups the AUC of DHA was higher than 0.7. The results of the curves were highly significant in patients affected by cancer, cardiopathies and HCV. The highest accuracy was highlighted in the groups with allergies, autoimmunity and thyroid diseases.

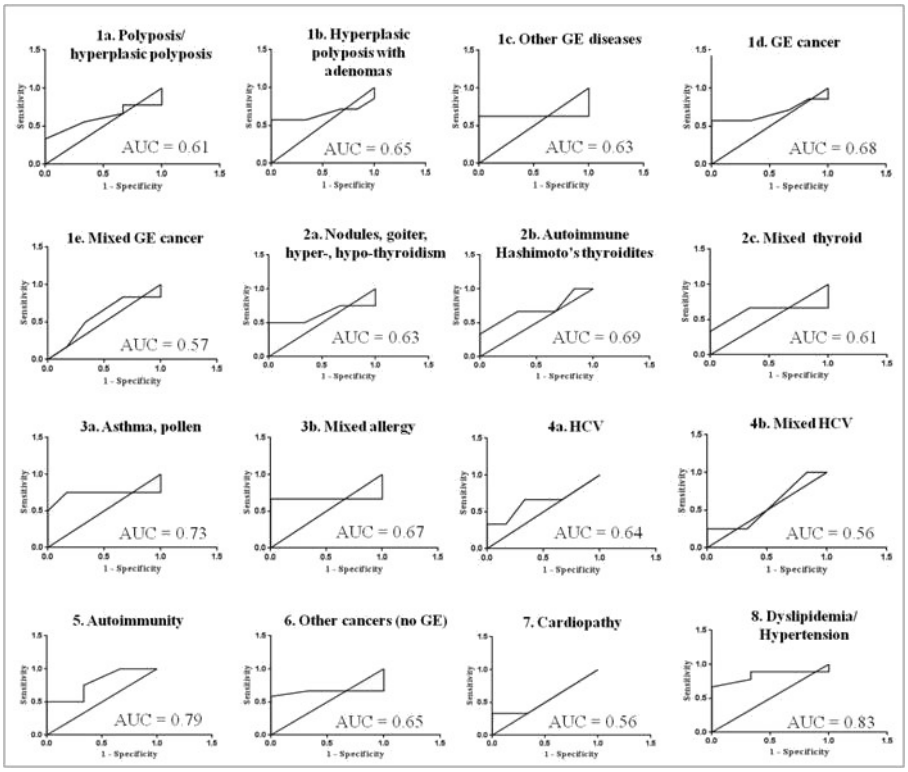

Figure 16: **ROC curves of DGLA values in patients with different pathologies.**

Since the ROC curve areas of DGLA were lower than 0.7 for almost all the groups, they were not particularly significant, except for groups of patients affected by allergy, autoimmunity and dyslipidemia/hypertension.

3.3.3 Mann Whitney U-test of PAR-PARP and fat profiles

Since in most groups the number of analized cases was limited, the non parametric Mann Whitney U-test was applied to confirm the results of ROC curves. In Table 10 the comparison of results of both tests is reported.

Table 10: **ROC curve and Mann Whitney (MW) test of PAR-PARP and some ω3 and ω6 PUFAs of analysed groups.**
In the table ROC curve areas and them *P* values, and Mann Whitney test *P* values are reported.

		PAR-PARP			EPA			DHA			DGLA		
		ROC curve		MW test	ROC curve		MW test	ROC curve		MW test	ROC curve		MW test
Group	Sub-group	AUC	*P*	MW *P*	AUC	*P*	MW *P*	AUC	*P*	MW *P*	AUC	*P*	MW *P*
1	1a	0.68	0.239	---	0.51	0.953	---	0.72	0.157	---	0.61	0.480	---
	1b	**1**	**0.003**	0.01	0.56	0.721	---	0.54	0.830	---	0.65	0.352	---
	1c	***0.79***	***0.071***	0.05	0.53	0.846	---	***0.77***	***0.093***	---	0.63	0.439	---
	1d	**1**	**0.003**	0.01	0.72	0.302	---	***0.82***	***0.054***	0.05	0.68	0.284	---
	1e	**0.86**	**0.037**	0.05	***0.81***	***0.078***	0.05	0.75	0.150	---	0.57	0.689	---
2	2a	0.54	0.830	---	0.65	0.456	---	**1**	**0.011**	0.01	0.63	0.523	---
	2b	**1**	**0.020**	0.01	0.64	0.519	---	0.83	0.121	---	0.69	0.366	---
	2c	***0.89***	***0.071***	0.05	***0.89***	***0.071***	---	0.83	0.121	---	0.61	0.606	---
3	3a	**1**	**0.011**	0.01	0.54	0.831	---	0.81	0.110	---	0.73	0.241	---
	3b	***0.89***	***0.071***	0.05	0.72	0.302	---	**1**	**0.020**	0.01	0.67	0.439	---
4	4a	***0.89***	***0.071***	0.05	0.67	0.439	---	***0.86***	***0.092***	0.05	0.64	0.519	---
	4b	**1**	**0.011**	0.01	0.69	0.337	---	**0.98**	**0.014**	0.01	0.56	0.749	---
5	---	0.79	0.136	---	0.71	0.286	---	**1**	**0.011**	0.01	0.79	0.136	---
6	---	**0.82**	**0.031**	0.05	**0.83**	**0.025**	0.05	***0.78***	***0.055***	---	0.65	0.301	---
7	---	**1**	**0.020**	0.01	0.78	0.197	---	0.83	0.121	---	0.56	0.796	---
8	---	**0.85**	**0.025**	0.05	0.70	0.195	---	0.57	0.637	---	**0.83**	**0.034**	0.05

AUC: ROC areas that were maximally significant (AUC= 0.8-1) are highlighted in bold; those with $P < 0.05$ are in bold italics.
MW test: only *P* values ≤ 0.1 are reported.

Mann Whitney test generally confirmed the significance of the ROC curves in groups and subgroups with *P* value at least below 0.1. In some cases the significance was very high, with very low *P* values (0.01). On the other hand, in few cases the data of ROC area were different from MW *P* for same PUFAs, in particular for EPA in subgroup 2c, and DHA in subgroup 1c and group 6.

3.3.4 Correlation between AA levels versus EPA, DHA, DGLA

The arachidonic acid (ω6) is the final product of the pro-inflammatory pathway. AA-derived eicosanoids have a role in regulating inflammatory mechanisms and their synthesis is influenced by the concentration of DGLA. As opposite EPA and DHA (ω3) are the products of the anti-inflammatory pathway. AA and EPA are formed by desaturases, specifically Δ5-desaturase, able to use both substrates (Figure 5).

The intake of ω3 leads to synthesize EPA and DHA rather than AA.

The correlation between AA and EPA, DHA, DGLA was evaluated in the different pathology groups with the Spearman's correlation test. The results are reported in Table 11.

Table 11: **Spearman's correlation test between AA versus EPA, DHA, DGLA.**

Group	Sub-group	AA vs. EPA	AA vs. DHA	AA vs. DGLA
1	1a	0.205	0.0	-0.103
	1b	0.272	0.357	0.204
	1c	0.277	0.687	-0.293
	1d	0.302	0.677	-0474
	1e	-0.366	-0.600	0.204
2	2a	-0.400	**-1**	-0.600
	2b	**1**	0.500	0.500
	2c	0.866	**1**	-0.500
3	3a	0.308	-0.718	-0513
	3b	-0.500	0.500	0.500
4	4a	**-1**	**-1**	0.500
	4b	0.800	**1**	0.103
5	---	-0.600	0.200	0.200
6	---	-0.205	0.559	0.552
7	---	-0.866	0.500	-0.500
8	---	0.391	0.695	-0120

Meaningful values (α = 0.05) are highlighted in bold.

The Spearman test indicated that the results of AA versus EPA were significant for subgroups 2b (Hashimoto' thyroiditis) and 4a (HCV). In the former a direct correlation ($\rho = 1$) was observed, whereas in the latter the correlation was inverse ($\rho = -1$). For AA versus DHA, in patients with mild thyroid disease (group 2a) and HCV (4a), AA was inversely correlated to DHA ($\rho = -1$). As opposite, a direct correlation ($\rho = 1$) was found in "mixed" thyroid diseases (group 2c) and "mixed" HCV (group 4b). However it was not possible to evaluate these results as both subgroups (group 2c, group 4b) enclosed patients suffering of other diseases, someones (hypertension, dislipidemy, cancer, adenomas, polyposis, cardiopathy) with ρ not significant.
DGLA and AA were not associated in any pathology.
It is worth noting that in subgroup 4a, both EPA and DHA inversely correlated with AA.

3.4 Correlation between PAR-PARP levels and PUFAs in the pathological groups

The non parametric Spearman's test was applied to evaluate a possible correlation between the levels of PAR-PARP and PUFAs in the pathology groups. The correlation coefficients were reported in Table 12.

Table 12: **Spearman's correlation coefficient of PAR-PARP versus PUFAs.**

Group	Sub-group	PAR-PARP vs. EPA	PAR-PARP vs. DHA	PAR-PARP vs. DGLA	PAR-PARP vs. AA
1	1a	0.204	0.133	-0.150	-0.035
	1b	0.0	-0.429	0.612	0.250
	1c	0.101	0.286	-0.421	0.547
	1d	0.041	0.070	-0.107	-0.146
	1e	-0.183	-0.314	0.495	-0.257
2	2a	0.400	**1**	0.600	**-1**
	2b	**1**	-0.500	-0.500	**1**
	2c	0.000	-0.500	**1**	-0.500
3	3a	-0.800	**1**	-0.200	-0.718
	3b	0.500	-0.500	**1**	0.500
4	4a	**-1**	**-1**	0.500	**1**
	4b	0.400	-0.200	-0.923	-0.200
5	---	-0.400	0.800	-0.800	0.400
6	---	0.234	-0.189	0.131	-0.294
7	---	-0.866	0.500	-0.500	**1**
8	---	-0.170	-0.174	-0.171	-0.267

Meaningful values are highlighted in bold (±1).

No association was found between the values of PARP and PUFAs for patients with gastroenteric diseases (groups 1, a-e), autoimmunity (group 5), no GE cancer (group 6) and dyslipidemia/hypertension (group 8).

PAR-PARP versus EPA were directly related in patients with Hashimoto's desease (group 2b), indirectly in those with HCV (group 4a).

A direct correlation between PAR-PARP values and DHA was found in people with mild thyroid diseases and allergy, whereas the association was inverse in patients with HCV.
As for PAR-PARP and DGLA a direct correlation was evidenced between the groups with “mixed” thyroid diseases and “mixed” HCV (groups 2c and 4b).
In patients with Hashimoto’s disease (group 2b), HCV (group 4a) and cardiopathies (group 7), an increase of AA corresponded to very high levels of PAR-PARP. An inverse correlation was observed between AA and PAR-PARP in subjects with mild thyroid disease (group 2a).

4. Discussion/Conclusions

This study started from both the knowledge that automodified PARPs (PAR-PARP), known as DNA break markers, are a direct measure of DNA damage extent, and that cell membrane lipid composition can be easily and accurately determined by fat profile. The research was carried on to study whether the tandem combination of the two tests could provide a non invasive tool to be used in the laboratory routine and able to depict the physio-pathological state of the cell.
At present, this work is the first study that analyse, in tandem, at molecular level, two very important features of the cell, i.e. the integrity of DNA (through PAR-PARP levels) and the cell membrane status (fat profile), defining their statistical significance.
Since diet is one of the most studied environmental factors in epigenetic changes (Waterland & Jirtle, 2003), there are increasing evidence that a correct quality of food intake and post-translational modification reactions (here namely poly(ADP-ribosyl)ation) might modulate deleterious processes as membrane lipid peroxidation and DNA damage respectively.
The central role of PARP1 and 2 as DNA damage sensors is to contribute repairing nucleic acid damage (Yelamos *et al.*, 2011). Both enzymes can be activated and automodified in response to DNA breaks by various patho-physiological conditions, including oxidative stress and inflammatory injury. Therefore, it is conceivable that their activity levels/expression in the cell nucleus can help in defining the physio-pathological state of the cell, offering a number of opportunities for therapeutic intervention in both cancer and other disease states (Morales *et al.*, 2014). Moreover, key roles for PARPs in metabolic stress and homeostasis have been proposed (Bai & Cantó, 2012). Insufficient or excessive nutrients induce cells to rearrange their metabolism, energy stores/expenditure. Such rearrangements heavily impact inflammation. Lifestyle and dietary changes may affect modulation of PARP function to improve health (Bai & Cantó, 2012). For instance, the NAD^+-consuming process is a consequence of PARP1 hyperactivation (Luo & Kraus, 2012; Kim *et al.*, 2014). The adaptation ability of cells towards metabolic stress drives the physiological or pathological states, as metabolic- or age-

related diseases (obesity, diabetes, cancer) (Wellen & Thompson, 2010).

As it regards PAR-PARP levels, our study confirmed their general meaning as DNA damage markers. Even before performing other more complex diagnostic analyses, and in accordance with data from literature, DNA damage is easily detectable by PAR-PARP measures.

The results of blood analyses clearly evidenced that in those patients with pathologies involving DNA damage, they were altered as a function of DNA break extent. Therefore the first conclusion is that as routine analysis, PAR-PARP measure could be predictive of possible diseases involving DNA breakage. Moreover, it is worth considering that, as stated above, increasing PARP automodification is strictly related to NAD^+ consumption. This reaction subtracts the pyridinic substrate from its coenzymatic function, and highly lowers its involvement in energy metabolism with deleterious consequences for ATP synthesis (Ying, 2006; Verdin, 2014). A relationship between PARP, DNA breakage, and niacin deficiency was reported previously (Pacher & Szabó, 2008). High PAR-PARP is an index of high consumption of NAD^+ and allows to evaluate a possible need of niacin supplementation. PAR-PARP levels were independent from BMI values. They showed to correlate with occurrence of diseases. Namely, the healthy subjects of the 84 examined people had low PAR-PARP levels indicating a physiological DNA damage that occurs during normal metabolic events such as DNA replication, transcription, etc. In case of diagnosis of serious diseases, high levels of PAR-PARP were confirmed. These results highlight the above mentioned role of PARP automodification that is used widely as DNA damage biomarker, for instance in the diagnosis of infiltrating inflammatory cells during stroke, myocardial and reperfusion injury, myocardial hypertrophy and heart failure, cardiomyopathies, circulatory shock, cardiovascular aging, atherosclerosis and vascular remodeling after injury, diabetic complications, and neurodegenerative disorders, since there is a wide literature about involvement of PARP in all these pathologies (Geraets *et al.*, 2007; Pacher & Szabó, 2007, 2008).

On the other hand, lipidomic profiling is an important tool to explore the impact of nutrition and metabolism (Chatgilialoglu & Ferreri, 2012). Lipids are derived preformed directly from the diet or

by *de novo* synthesis from simple precursors. Variations in lifestyle and genetics influence the enzymatic activities involved in their metabolism. Dysregulation of lipid metabolism results in diseases. Moreover, cell membrane is a sensor that changes and adapts continuously to metabolic stimuli and to the external environment (diet, stress, physical and chemical agents), and its properties are regulated by fatty acid composition.

The rationale of BMI analysis (first approach) arose from the observation that overweight and obesity have recently reached vast proportions worldwide, representing one of the main historical metabolic risk factors. The onset of these conditions of excess weight has remarkable effects on health status with an increased risk of inflammatory and cardiovascular diseases and some cancers. In this context, the first evaluation criterion was the comparison of the results of the analyses with both anthropometric measures (body mass index) and any clinical state of subjects undergoing the survey. As a result, in this study, the content of all FA categories was altered in any BMI groups.

The evidenced excess of SFA was independent from anthropometric data. Even most of normal weight people had high SFA levels. Indeed SFA excess was an index of increased membrane rigidity, with consequent possible altered permeability and functionality. Moreover, it might indicate alterations in any enzymatic step of FA metabolism requiring further attention. In fact, in some cases the increase of MUFA like palmitoleic and vaccenic acids suggests the activation of the metabolic conversion of SFA into MUFA, to balance their excess and to reduce possible metabolic and cardiovascular risks (Baritaki *et al.*, 2007; Ferreri, 2009; Chatgilialoglu *et al.*, 2014). The knowledge of the actual status of a subject allows defining a dietary regimen to equilibrate the membrane lipids and to support specific therapies. In synthesis, these results at least allow correcting the quality of food, limiting the intake of nutritional sources of SFA, in order to re-establish the right content of these FA category.

A high unbalance was measured for ω6/ω3 PUFA too. In some cases, there was a net increase of arachidonic acid, a precursor of many pro-inflammatory compounds, compared to the expected normal ranges.

Mostly a high reduction of ω3 PUFA was observed. Due to the anti-inflammatory value of these FA, their deficiency indicated a low anti-oxidant defense ability of cells, and a high risk of inflammation.

A behavior similar to that evidenced for PAR-PARP levels.
Statistical analyses did not evidence any correlation between the two tests in BMI patient groups.
As a conclusion of the results of the first approach, the two analyses give information about DNA damage involvement (PAR-PARP levels), and on the balance of membrane lipids.

The results reported for the second approach (the two tests versus pathologies) gave indications coherent with the data described in literature.
In Table 11 the results reveal an indirect correlation between DHA and AA in patients affected by thyroid diseases. It has been widely demonstrated that omega 3 FAs regulate the levels of the thyroxine hormone, T4 (Taraghijou *et al.*, 2012). This thyroid hormone plays a role in several body functions, including growth and metabolism. High levels of DHA, probably due to a therapeutic treatment ongoing, balance the pro-inflammatory effects of AA, stayed at low concentrations.
In patients with Hashimoto's hypothyroidism low levels of AA correspond to a decrease of EPA. In fact, it is known that this condition is characterized by a reduction of the AA levels (Guerriero *et al.*, 1999). Moreover, the alteration of thyroid hormone synthesis is explained by the decrease of EPA.
In group 4a (HCV) an increase of arachidonic acid, the inflammatory mediator, corresponded to a decrease in the levels of EPA and DHA. It is known that the excess of AA promotes the synthesis of prostanoids of group 2 (PGD_2, PGE_2, PGF_2, PGI_2, TXA_2), leukotrienes of series 4 and lipoxins that favor the inflammatory effects (Ferreri & Chatgilialoglu, 2012).
Evidence in the literature suggests that PUFAs can have both positive and negative effects on the host antiviral response (Anderson & Fritsche, 2002). PUFAs have the ability to inhibit HCV RNA replication at physiologically relevant concentrations (Das, 2008). AA, EPA and DHA had the capacity to exert anti-HCV activities using an HCV subgenomic RNA replicon system, that is independent of their ability to suppress lipogenic gene expression (Kapadia & Chisari, 2005; Liu *et al.*, 2010). The anti-HCV action of PUFAs is due to the formation of significant products of lipid peroxidation that

occurs only in cells where the viral replication is activated (Huang *et al.*, 2007).
In patients with chronic hepatitis C the results showed a decrease of EPA and DHA, important modulators of a host's inflammatory/immune responses (Chapkin *et al.*, 2009). This decrease is probably due to antiviral therapy ongoing. Indeed, it is known that patients undergoing IFN-a and ribavirin treatment have markedly higher AA and decreased EPA levels in peripheral blood mononuclear cells (Hino *et al.*, 2006).

In table 12 the patients affected by thyroid diseases (group 2a) had a physiological level of PAR-PARP, as shown in the ROC curve (AUC= 0.54). Low values of PAR-PARP were associated to increased DHA.
DHA balances the inflammatory effects of AA, and promotes the production of protective substances, such as neuroprotectin, which prevents inflammation in the neuronal tissue (Ferreri & Chatgilialoglu, 2012).
Hashimoto's desease (group 2b) caused a hyperactivation of PARP (AUC= 1), directly related to the increase of AA and EPA. Therefore the inflammatory effects of AA are unbalanced from the increase EPA, probably caused by the current treatment.
In patients with allergy an increase of PAR-PARP corresponded to a increase in the levels of DHA and DGLA (groups 3a and 3b). Different studies suggested the involvement of PARP1 in the pathogenesis of allergic airway inflammation, such as asthma and allergic rhinitis (Ozaydin *et al.*, 2014).
The activation of PARP results in an up-regulation of pro-inflammatory gene expression countered by the action of ω3 PUFAs, that decrease the production of inflammatory mediators (eicosanoids, cytokines, and reactive oxygen species) and the expression of adhesion molecules. EPA and DHA suppress PGD_2, IL-13 and IL-4 secretion as well as ROS generation most effectively (van den Elsen *et al.*, 2013). In this case DGLA has an anti-inflammatory effect because inhibits phospholipase A_2, blocking the synthesis of AA and other pro-inflammatory mediators and releases anti-inflammatory PGE_1.
In patients with HCV (group 4a) PAR-PARP levels were directly correlated to those of the AA, inversely to the values of EPA and DHA. Higher levels of AA compared to those of EPA and DHA lead

to an overproduction of the pro-inflammatory prostaglandins of the ω6 series and cytokines.
The results of Sperman's test showed a direct correlation between PAR-PARP and AA levels (group 7). PARP1 activation plays a role in the pathogenesis of various cardiovascular and inflammatory diseases (Szabó, 2005). The potential role of PARP2 in cardiovascular system was determined too. PARP2 deletion protected cardiomyocytes from hypertrophy, which may be attributed to activation of the key member of sirtuins (SIRT) family, SIRT1 (Geng *et al.*, 2013).
The inflammatory condition promotes the activation of PARP and simultaneously causes an alteration of the ω6/ω3 ratio. Different evidence in literature confirm the excessive amounts of ω6 polyunsaturated fatty acids (PUFA) and a very high ω6/ω3 ratio the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases (Simopoulos, 2008).
Recent findings suggest that higher plasma levels of ω6 PUFAs, mainly AA, were associated with decreased plasma levels of serum pro-inflammatory markers, particularly interleukin-6 and interleukin-1 receptor antagonist, and increased levels of anti-inflammatory markers, particularly transforming growth factor-β. Therefore, AA and ω3 PUFAs may modulate the inflammatory response by acting both on the pro-inflammatory and anti-inflammatory arms of the cytokine network (Ferrucci *et al.*, 2006; Harris *et al.*, 2009).

In conclusion, the two analyses in tandem seem to contribute to draw at least diagnostic clinical pictures to support therapeutic and/or nutraceutical approaches.

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