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Coordinator: Prof. Lorenza Trabalzini

Therapeutic potential of the novel compound N-acetylcysteine ethyl ester (NACET) in modulate plasma thiols and homocysteine levels

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TUTOR Prof. Daniela Giustarini

(co-TUTOR) Prof. Ranieri Rossi

PhD STUDENT

Sante Colella

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Abstract

Hyperhomocysteinemia (HH) is a condition characterized by a high plasma concentration of homocysteine (HCys), a cysteine metabolite (Cys), which has proven to be a risk factor for a wide range of cardiovascular, neurological, and metabolic diseases. Among the variants of HH, the mild form (mHH) represents an important subgroup that, although less severe, can still significantly contribute to the risk of cardiovascular and neurodegenerative diseases. One of the mechanisms by which HH can exert its harmful effects is the reduction of glutathione levels (GSH), an important endogenous antioxidant mechanism involved in protecting cells from oxidative damage. The use of therapeutic strategies based on the administration of B vitamins to reduce circulating levels of HCys has produced conflicting results on their real effectiveness. Therefore, the attention of the scientific community has focused on new more decisive alternatives.

In this current work, our initial aim was to assess plasma levels of HCys in a meticulously chosen cohort, while diligently mitigating potential confounding variables. Specifically, we restricted sample collection to a three-month timeframe (April-June) to minimize seasonal variations in fruit and vegetable availability that could influence outcomes. Additionally, we limited the age range of participants to 20 years, recognizing the well-established impact of age on HCys levels, and implemented selection criteria based on dietary habits, alcohol consumption, and other pertinent factors. Stemming from the observation that an increase in plasmatic low molecular weight thiol is associated with a decrease in circulating homocysteine, we posited the hypothesis that homocysteine itself may not be inherently detrimental to the cardiovascular system, but rather serves as a biomarker indicating underlying physiological irregularities. Our results showed an inverse correlation between circulating LMW-SH and HCys levels.

Through other *in vitro* studies, a series of experiments have been carried out to highlight cellular and/or tissue participation in GSH efflux. Therefore, we have carried out comparative studies with several pro-Cys drugs to evaluate and analyze their effectiveness in increasing intracellular synthesis and GSH efflux.

In this context, the administration of N-acetylcysteine ethyl ester (NACET), a pro-Cys drug, emerges as a promising strategy to modulate GSH levels and reduce the novel effects of HH lowering the plasmatic HCys levels.

This paper aims to examine the role of NACET in modulating GSH levels through a series of experiments aimed at identifying the parameters that are used to describe a human's thiolome, this part was performed on blood samples from a group of healthy subjects.
Our attention then focused on an *in vitro* study in which blood samples were subjected to oxidative stimuli to analyze other biomarkers of oxidative stress (malondialdehyde and protein carbonyls).

Finally, studies have been carried out on how NACET can influence the synthesis and release of GSH and modulate the levels of circulating plasma HCys through *in vitro* experiments on cell cultures or blood samples comparing its effectiveness with other thiol compounds.

List of abbreviations	
A375	Human melanoma-derived cells
A549	Human lung carcinoma-derived cells
ADMA	Asymmetric dimethylarginine
ADT	Anethole dithiolethione
AGE	Advanced glycation end-products
ALE	Advanced lipoxidation end-products
ARPE-19	Retinal pigment epithelial cells
ATP	Adenosine triphosphate
BMI	Body mass index
CBS	Cystathionine-β-synthase
Cys	Cysteine
Cys34	Cys in position 34 on the aminoacid chain in HSA
CysGly/CG	Cysteinylglycine
CysGlySS	Cysteinylglycine disulfide
CysGlySSP	CysGly mixed disulfide with protein
CysGlySSP	γ-GluCys mixed disulfide with protein
CySS	Cystine
CySSG	Cysteine-glutathione mixed disulfide
CySSP	Cysteinylated protein
Cysα13	Cys in position 13 on the aminoacid chain in rad Hb
Cysβ125	Cys in position 125 on the aminoacid chain in rat Hb
DMEM	Dulbecco's Modified Eagle Medium
DMSA	Dimercaptosuccinic acid
DNPH	2,4-Dinitrophenylhydrazine
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	Dithiothreitol
EBM-2	Endothelial Cell Growth Basal Medium-2
ECL	Enhanced chemiluminescent
FBS	Fetal bovine serum
GCS	γ-glutamyl cysteine synthetase
GlyNAC	NAC and glycine compound
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSH-EE	Glutathione ethyl ester
GSHS	GSH synthetase
GSSG	Glutathione disulfide
GSSP	S-glutathionylated protein
Hb	Hemoglobin
Hb-SSG	S-glutathionylated hemoglobin
Hb-SSP	Mixed disulfide with hemoglobin
HCys	Homocysteine
HCySS	Homocystine
HCySSP	Homocysteinylated protein
HDL	High density lipoprotein
HEK293	Human embryonic kidney-derived cells

HeLa	Henrietta Lacks cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HH	Hyperhomocysteinemia
HIV	Human immunodeficiency virus
HMW-SH	High molecular mass thiol
HMW-SS	High molecular mass disulfide
HPLC	High performance liquid chromatography
HSA	Human serum albumin
HSA-HCys	Homocysteinylated human serum albumin
HSA-SH	Mercaptoalbumin
HUVEC	Human Umbilical Vein Endothelial Cells
IMR90	Human embryonic lung-derived fibroblast
K_3EDTA	Tripotassium ethylenediaminetetraacetic acid
LDL	Low density lipoprotein
LMW-SH	Low molecular mass thiol
LMW-SS	Low molecular mass disulfide
mBrB	Monobromobimane
MDA	Malonyldialdehyde
MESNA	sodium 2-mercaptoethane sulfonate
MetOH	Methanol
mHH	Mild hyperhomocysteinemia
MP-SG	S-glutathionylated RBC membrane protein
MP-SX	S-thiolated RBC membrane protein
MRP	Multidrug resistance-associated protein
MTHFR	Methylenetetrahydrofolate reductase
NAC	N-acetylcysteine
NACET	N-acetylcisteine ethyl ester
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-etylmaleimide
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drug
-OH	Alcohol group
OTC	2-oxothiazolidine-4-carboxylic acid
PBS	Phosphate buffer saline
PCO	Protein carbonyls
PMN	Polymorphonuclear cell
PSH	Protein thiol
PSSG	S-glutathionylated protein
PSSP	Protein disulfide
PTI	Protein thiolation index
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
RNS	Reactive nitrogen species
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
RSSP	Protein mixed disulfide
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

-SH	Sulfhydryl group
SOD	Superoxide dismutase
SOH	Sulfenic acid
SS	Disulfide
TBA	Thiobarbituric acid
t-BOOH	Tert-butyl hydroperoxide
TCA	Trichloroacetic acid
tCys	Total cysteine
tCysGly	Total cysteinylglycine
tGSH	Total glutathione
tHCys	Total homocysteine
TSP	Transsulfuration pathway
UV-Vis	Ultra-violet visible
γ-GluCys	γ-glutamylcysteine
γ-GluCySS	γ-glutamylcysteine disulfide
γ-GT	γ-glutamyl transpeptidase

Introduction

One of the greatest evolutionary achievements that living beings on Earth have experienced is the transition to an oxygenated atmosphere, and although it is the basis of life, the 21% oxygen content in the air can bring certain problems. During normal processes of cell metabolism for energy production, for example, oxygen molecules may undergo an incomplete reduction, generating reactive oxygen species (ROS) that include superoxide anions and hydrogen peroxide (1, 2). ROS production occurs when, during cellular respiration processes, electrons escape from the functional groups of electron chain transport systems, thus reducing molecular oxygen to superoxide anion. Not only that, ROS production also occurs enzymatically in various cell organelles (such as lysosomes and peroxisomes) (3) and is functional for normal cell metabolism. Moreover, ROS are produced both by macrophages and neutrophils (by their function against pathogens) and by non-phagocytic cells (4). The production of superoxide anion is quickly followed by its conversion to hydrogen peroxide by the enzyme superoxide dismutase (SOD). Some superoxide anions, however, can react with nitric oxide to form peroxynitrite, a high-reactive nitrogen species (RNS) (5). Finally, hydrogen peroxide is also capable of reacting with metal ions (iron and copper) to form very reactive and short-lived compounds called hydroxyl radicals via what is called the Fenton reaction (6). RONS production, able to cause irreversible damage by reacting with DNA, proteins, and lipids (7), is however accompanied by an effective antioxidant system that allows the organism to survive (8). Mammalian cells function in a reducing environment where the production and management of small amounts of RONS are used as a signaling mechanism. Hydrogen peroxide is both an important signal of intracellular transduction of cell growth and a secondary messenger for the activation of different cellular processes such as gene expression, for example. The first definition of oxidative stress described it as an "imbalance between pro-oxidants and anti-oxidants in favor of the former" (9). However, this definition has become obsolete because, with the increase of publications in the field of oxidative stress, it has been seen that neither the increase of oxidizing species (whose production and use by cells is physiological) nor the decrease of antioxidants, alone, can generate oxidative stress. A more appropriate description of oxidative stress was subsequently developed as "a disturbance in the pro-oxidant/anti-oxidant balance in favor of the former, leading to potential damage" (10) where there is an increase in oxidizing species levels (with probable decrease of anti-

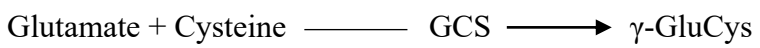
oxidants ones) and oxidative damage to cellular biomolecules and structures. Antioxidants are molecules that act by counteracting the excessive formation of oxidizing species or inhibiting their reaction with cellular structures or biomolecules. Their action takes place either by enzymatic (SOD, catalase, glutathione peroxidase, and reductase) or in a non-enzymatic way through the action, for example, of the vitamins α-tocopherol (vitamin E), β-carotene (vitamin A) and ascorbic acid (vitamin C) or the tripeptide glutathione (GSH). The greatest buffering action at the cellular level against RONS is constituted by cysteinyl thiols which, taken as a whole, provide a thiols range concentration of about 10-30 mM (11). Moreover, oxidative stress can induce reversible and/or irreversible changes to proteins sensitive to oxidation phenomena that can lead to partial or total loss of protein function (12). The reversible modifications, very often, involve residues of cysteine (Cys) and can protect proteins from irreversible damage, modulate their action, being involved in redox signaling or an early response to mild oxidative stress (13, 14). Irreversible changes (such as protein-protein cross-links or protein carbonylation) can lead to total loss of protein function, unfolding, protein degradation through cellular systems, or accumulation of protein residues in intra and extracellular sites (as observed in some age-related neurodegenerative diseases) (15, 16). Traces of oxidative damage can be found in all known diseases, but there is no clear correlation between disease and damage from RONS for most pathological conditions. In some disorders, however, oxidative damage plays a major role in the pathogenesis of the disease. For example, diseases behind intestinal malabsorption can lead to a lower intake of selenium or vitamin E (17, 18). Moreover, disorders related to altered metal homeostasis (such as Wilson's disease or thalassemia) are strongly accompanied by oxidative stress due to the action that metals have in the production of RONS (19, 20). Exposure to exogenous factors (such as ionizing radiation and ultraviolet radiation) or high doses of air pollutants (such as ozone) also favor the generation of oxidizing species which, in these cases, are more related to inflammatory diseases of the respiratory airways (21). In other pathological conditions, the connection between oxidative damage and the development of the disease due to an increase in oxidizing species can be hypothesized although there are some examples where it is plausible to assume it. Inflammation, for example, is one of those physiological processes in which the formation of RONS, when not properly regulated, can lead to oxidative damage. Activated and recruited neutrophils in inflammatory sites can kill bacteria through the production of RONS but, at the same time, can damage adjacent

tissues (22) if the cells recruited are many and/or the inflammatory process lasts for too long. Inflammatory processes that promote the oxidation of lipids and proteins also play a pivotal role in the pathogenesis of atherosclerosis. The pathology begins when damage in the endothelium is formed, and macrophages recruited on-site begin to produce RONS and, as a result, oxidative damage. Low density lipoproteins (LDL) are internalized by macrophages and allow the release of molecules that can worsen endothelial injury. In addition, LDL can act directly in the inflammatory cell pool by modulating its number (23). Finally, the oxidizing species produced by macrophage myeloperoxidases can inhibit the protective action carried out by high density lipoproteins (HDL) (24). During an ischemic tissue phenomenon, in which oxygen transport fails, tissues can be damaged or killed. Re-oxygenation, in these cases, can be an even more dangerous phenomenon when the tissue has not yet been irreversibly damaged. The lack of oxygen causes a reduction in the production of adenosine triphosphate (ATP), the pH drops, the iron stored inside the cells can be released and the mitochondrial "leakage" of the electron transport chain can increase (increased superoxide production) (25). The released iron can also participate in other reactions within the cell: the ischemic phenomenon increases the cellular content of an enzyme (xanthine oxidase) (26) capable of forming superoxide and hydrogen peroxide from hypoxanthine. These reactive species, along with iron, can lead to the production of the hydroxyl radical increasing the damage. Oxidative stress is also present in the progression of diabetes mellitus and all those vascular complications that result from it (27). Altered blood glucose values can induce oxidative stress through, for example, auto-oxidation of glucose, formation of advanced glycation and lipoxidation end-products (AGEs/ALEs), alteration of redox balance due to increased production of RONS, and glycation of enzymes and cellular protein structures (28). Aging is considered the result of an accumulation of damage induced by mitochondrial RONS (29). Moreover, antioxidant defenses do not always effectively protect biomolecules and structures from aging-related oxidative damage (30). Although it is accepted that mitochondrial structure and DNA degeneration are responsible for age-dependent damage, studies to reduce or inhibit RONS production have shown no improvement (31).

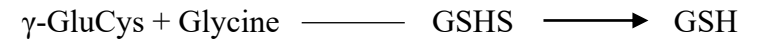
1.1-Thiols and disulfides

Among all the antioxidant systems available in the cell, enzymatic and not, thiols represent one of the most important due to their chemical properties both to buffer the natural (and pathological) production of RONS and for the regulation of intra and extracellular redox. The great reactivity of thiols is given by the sulfur atom contained in their structure; this transition metal polarizable and capable of adopting different oxidation states is at the center of a complex network of sulfur-based reactions. The sulfhydryl group of thiols (-SH), also known as mercaptans or thioalcohols, is analogous to the hydroxyl group present in alcohols (-OH), the replacement of oxygen with sulfur, however, allows thiols to have unique chemical and biological behaviors. Physiological thiols can undergo almost all known chemical reactions of biological interest such as addition and oxidation. Thiol oxidation can generate reversible modifications, namely disulfides (SS) and sulfenic acid (SOH) or sulfinic and sulfonic acids which instead, are irreversible changes that result as damaging by-products for biomolecules. In vivo thiols can be categorized into low molecular mass thiols (LMW-SH) and high molecular weight (or protein) thiols (HMW-SH, PSH); the most studied LMW-SH for their biological interest are cysteine (Cys), homocysteine (HCys), γ-glutamyl cysteine (γ-GluCys) and glutathione (GSH). Of the twenty amino acids known both Cys and methionine contain a sulfur atom but only Cys contain a sulfhydryl group. In addition, despite its low intracellular concentration, Cys is an amino acid of primary importance as it is essential for protein synthesis and a limiting factor for the synthesis of GSH. HCys instead plays a pivotal role in the metabolism of methionine, its action occurs in the transsulfuration pathway (TSP); in TSP, HCys leads to the formation, in a metabolic pathway, of Cys, taurine, and other sulphate compounds and, in another pathway, to the re-methylation of HCys to form methionine. There are substantial differences between tissue and blood HCys levels, in fact the concentrations of the amino acid are much higher in the extracellular environment than in the intracellular one; cellular HCys is catabolized and exported to plasma where it is metabolized mainly by the kidneys, and, in small part, excreted with urine (32). As well as Cys and HCys, despite their involvement in the GSH synthesis, also γ-GluCys has low tissue concentrations. Finally, the tripeptide γ-glutamyl-cysteinyl-glycine (glutathione, GSH) is the most abundant cellular thiol (33) with a concentration ranging from 2-10 mM (34); in the cell, most of the GSH is localized in the cytosol. GSH synthesis is catalyzed

by the joint action of two enzymes starting from the constituents: glutamate, cysteine, and glycine. GSH is produced constitutively in all cell types although hepatocytes are the largest producers and exporters of GSH. The first enzyme aimed at the synthesis of GSH is γ-glutamyl cysteine synthetase (GCS) where the γ-carboxyl group of glutamate reacts with the amino group of cysteine forming a peptidic γ-linkage (which protects GSH from the hydrolytic action of intracellular peptidases) resulting in the formation of γ-GluCys. Mammalian GCS is a heterodimer that has a heavier catalytic part (73 kDa) and a lighter regulating part of 31 kDa. The larger complex accommodates the binding sites for the substrates while the lighter complex modulates the affinity of the heavy part for substrates and inhibitors. GCS is a rate-controlling enzyme in the de novo synthesis of GSH, the synthesis of this enzyme also increases when the cell undergoes a series of phenomena that cause a depletion of intracellular GSH reserves (such as oxidative stress or inflammation, for example). The first step in the synthesis of GSH is of particular importance as the activity of the enzyme is subject to non-allosteric inhibition by GSH concentration and Cys levels (limiting factor).



The second enzyme for GSH synthesis is the GSH synthetase (GSHS) a 52 kDa homodimer/subunit and an allosteric enzyme that binds γ-glutamyl substrates.



GSH degradation occurs in the extracellular environment by enzymes such as γ-glutamyl transpeptidase (γ-GT) and dipeptidases that form sequentially first cysteinyl glycine (CysGly) and then Cys. The basic components of GSH formed by the action of these ectoenzymes are then transported back into the cells for the de novo synthesis of GSH ensuring a very rapid turnover (synthesis and export). GSH is almost ubiquitous in all organs, the highest concentrations are found in the liver tissue which also represents the main exporter of GSH where, Cys coming from food or TSP, is converted to tripeptide. GSH performs several biological functions through its cysteinyl portion; it acts as a radical scavenger and reducing agent because of the sulfhydryl group present in its structure. And, although the thiol portion of GSH has a fairly high pKa value (8.9-9.4) (35) that indicates low intracellular reactivity, its antioxidant action is based on

enzymatic reactions involving it, efficient recycling patterns, and high intracellular concentrations. Finally, GSH participates in detoxification reactions by conjugating to the electrophilic center of xenobiotics (or their metabolites) or endogenous molecules through the action of enzymes called GSH S-transferase (36). All intracellular LMW-SH mentioned so far (GSH, Cys, HCys, γ-GluCys) participate in different pathways that, in the end, are all metabolically related. HCys regenerates in Cys through TSP, the latter is used, together with glutamate, for the formation of γ-GluCys which is a substrate for the synthesis of GSH that is used as a cofactor in reduction reactions or extracellularly degraded in its base components to be re-synthesized *de novo* within the cell.

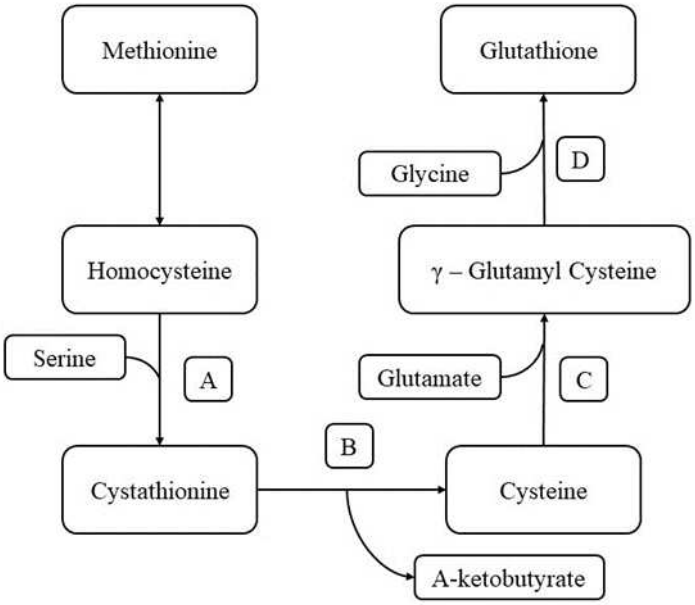


Figure 1. **Schematic correlation between pathways that correlate LMW-SH. A**. Cystathionine β – synthase. B. Cystathionase. C. γ – glutamyl cysteine synthetase. D. Glutathione synthetase.

In the cell, besides the LMW-SH, there are also PSH or HMW-SH that, compared to the former, have a higher concentration. As mentioned above, Cys is important for both GSH production and protein synthesis. This amino acid can be part of the polypeptide chain of a protein and, depending on its position can perform a different function. PSH are all those proteins that contain a free Cys residue and, therefore, a functional sulfhydryl group (-SH). The action of the free thiol portion of a PSH can be performed in several ways. Some PSHs are enzymes, and the thiol portion is the catalytic center of the enzyme that is often involved in redox reactions, nucleophilic attacks, and metal regulation within the cell. In addition, the redox state of the thiol portion affects both

enzymatic activity and substrate binding. PSH redox state also plays a role as signalling mechanism: reversible reduction and oxidation processes can regulate protein function, protein interaction, and gene expression. HMW-SH, together with LMW-SH, can act as antioxidants protecting the cell from oxidative stress, the action is performed by acting both as a buffer system for oxidizing species and by repairing proteins damaged by oxidative stress, thus helping to maintain the homeostasis redox of the cell. Finally, PSHs can participate in cell signalling pathways by forming disulfide bonds with specific proteins and leading to the activation or inhibition of signalling cascades. The most peculiar property of the -SH group in biological systems is the readily oxidizability to form disulfides (SS). This group of molecules exists in biological systems in several forms, i.e. low molecular (LMW-SS) and high molecular mass disulfides (HMW-SS). They can be symmetrical when the two components are the same, as in the case of cystine (CySS) or glutathione disulfide (GSSG), or asymmetric when the two molecules are different. Given the high concentration of GSH within cells, GSSG is the major product of an overproduction of RONS, with the GSH/GSSG parameter being frequently investigated as a biomarker of oxidative stress. HMW-SS, instead, includes both interprotein disulfides and mixed disulfides with LMW-SH. Cys residues within proteins are important during the folding of the maturing protein for the formation of the tertiary structure; disulfide bridges guarantee the final functionality of the protein. Disulfide bridge formation within proteins is a process that occurs during the maturation of the protein in specific compartments (for example, the endoplasmic reticulum) by different enzymes such as oxidoreductases and thiol oxidases. As mentioned above, in addition to having the free thiol portion, PSHs can take other oxidation states (forms of sulfenic, sulfinic, or sulfonic acids) including the formation of mixed disulfides with LMW-SH reacting with HCys, CysGly, Cys, and GSH that are called S-thiolated proteins. Thiolation/de-thiolation processes are guaranteed by the accessibility that the cysteinyl residue has to redox reactions inside the protein. Both the three-dimensional structure and the reactivity of Cys in the polypeptide chain are determining factors for the formation of mixed disulfides to take place. At the cellular level the most abundant LMW-SH are GSH and Cys therefore S-glutathionylated and S-cysteinylated proteins will be the most present mixed disulfides. Protein S-glutathionylation can be considered a post-translational modification that also occurs under physiological conditions, which suggests that it is a related process in cell signaling and redox regulation of protein functions (37). In addition, protein S-

glutathionylation could also be involved in GSH storage processes and thus the protection of cysteinyl residues from oxidative stress by masking them with GSH. Adding a GSH molecule to the cysteinyl portions of proteins, even if it is a reversible modification, can inactivate the protein in case the -SH functional group is the functionally active part of the peptide. The modification, moreover, can both change the peptide conformationally and change its charge because, by the GSH glutamate residue, a negative charge is introduced to the molecule. The formation of mixed disulfides with proteins involving GSH can occur due to changes in the intracellular GSH/GSSG ratio through a thiol/disulfide exchange reaction between the -SH group of the protein and the GSSG; the reaction not only removes GSSG but also regenerates GSH helping to restore the redox physiological balance.

$$PSH + GSSG \longrightarrow PSSG + GSH$$

There are also other mechanisms through which GSH adducts can be added to PSH, many cells can export GSSG molecules in response to cellular increases thereof and, therefore, under physiological conditions, hardly ever have large intracellular increases of GSSG. An example is the partial oxidation of the cysteinyl residue of PSH or GSH by a radical reaction to form a thiyl radical that will then react with, respectively, GSH or PSH to give an S-glutathionylated protein.

$$PS^{\circ} + GS^{-} + O_2 \longrightarrow PSSG + O_2^{\circ -}$$

$$PS^{-} + GS^{\circ} + O_2 \longrightarrow PSSG + O_2^{\circ -}$$

It should be noted that this type of modification to thiol proteins is important as a mechanism of cell signalling but also as it is reversible and, the reverse process of de-glutathionylation, can take place either by exchanges of thiol/ disulfide with GSH (once the redox balance of the GSH/GSSH ratio has been restored) or, more frequently, through oxidoreductase catalysis (37). Below is a table that summarizes the intracellular thiols of greatest biological interest.

Intracellular Thiols	
Reduced Form	**Disulfide Form**
Low molecular mass thiols: - Glutathione - Cysteine - Homocysteine - Cysteinyl glycine - γ – Glutamyl cysteine	*Low molecular mass disulfides*: - Glutathione disulfide - Cystine - Homocystine
High molecular mass thiols: - Protein thiols	*High molecular mass disulfides*: - Interprotein disulfide - Intraprotein disulfide - Protein mixed disulfide with LMW-SH
Other forms of protein thiols modifications: - Protein thiol oxidized to sulfenic acid - Protein thiol oxidized to sulfonic acid - Protein thiol oxidized to sulfinic acid	

Table 1. **List of all intracellular thiols and disulfides of physiological interest**.

1.2-Blood thiols: the difference between corpuscular part and plasma

Of considerable biological interest is also the picture of the thiols and disulfides present at the extracellular level and, more specifically, at the blood level. The main difference between blood and the intracellular/tissue compartment is that the latter is a more reducing environment, while the blood is a more "pro-oxidant" environment that affects all the thiol species that are present in it. The proportion of thiols in reduced form is much higher at the tissue level than in blood which, on the other hand, presents mostly disulfides. Before proceeding with the classification of thiols and disulfides and then highlighting the differences that there are with the tissue environment, it is good to divide the blood into its two components: a cellular part, corpuscular, consisting of blood cells, including red blood cells, white blood cells and platelets, and a liquid component consisting of plasma. Regarding the corpuscular component, red blood cells (RBC) are the most studied subjects because, the analysis and quantification of the thiols present in them, are more accurate and less subject to artifacts and operator-dependent oxidations. The LMW-SH most present at the level of erythrocytes is GSH (about 3 mM for RBC) although there are also Cys, HCys, and γ-GluCys but in lower concentrations (38). The RBCs can synthesize GSH as they possess, within them, all the enzyme complexes that are involved in the synthesis of GSH. Not only that, the erythrocyte production of GSH contributes to the tripeptide pool together with other specialized tissues (such as hepatocytes); the synthesis is also regulated according to the physiological/pathological disturbances of the GSH/GSSG ratio, in erythrocytes the reduced form of GSH constitutes about 99.8% of the total and only 0.1% is in the GSSG form that makes it the most present disulfide being actively exported by RBC in case of sudden increases (38). As for disulfides mixed with proteins, the remaining 0.05% of GSH of RBC is involved in the phenomena of S-glutathionylation with hemoglobin. Glutathione hemoglobin (Hb-SSG) could also be a useful marker of oxidative stress because, as described above, the phenomenon of S-glutathionylation, is also highly regulated by the oxidizing conditions of the environment in which the protein is located (39). As for the LMW-SH within white blood cells, the GSH is the most present (2-4 mM per cell) (40) although the contribution that these cells can have to the GSH total pool of the body is lower because, the leukocytes, are much less than the erythrocytes. A very important thiol/disulfide pool, in the extracellular compartment, is represented by the plasma; this is involved in cell signaling processes, antioxidant defense, metal

homeostasis, and protein regulation. Plasma thiols have antioxidant properties and interface with the thiol/disulfide composition of PSHs through thiol/disulfide exchange. The reduced/oxidized species ratios of the various thiols are not in equilibrium in the plasma, this allows both a rapid and dynamic regulation of redox-based signaling processes and a buffer system for oxidative damage mediated by non-radical species. Despite the antioxidant species present in the plasma, it cannot be considered an "antioxidant fluid", LMW-SH in reduced form has relatively low concentrations. An increasing number of studies have also shown how the plasma thiol/disulfide pool of LMW-SH (Cys, HCys, CysGly, γ-GluCys, and GSH) is involved in oxidative reactions and antioxidant defense during the pathogenesis and the course of numerous pathologies. In plasma, LMW-SH are present at very low concentrations, and, taken together, they have a total concentration of about 12-20 μM (40). Starting from Cys, compared to the tissues where it has a much lower concentration, the plasma is the most present LMW-SH with levels reaching approximately 10 μM (free Cys) and is involved in the balance processes of inter-organ GSH production and protein synthesis. In plasma, HCys levels are of particular biological interest as they are closely interconnected with the course of cardiovascular disease and/or atherosclerosis. In a healthy individual, the proportion of HCys present in the plasma is about 0.17-0.32 μM. CysGly has slightly higher values than HCys (2-2.9 μM) while γ-GluCys has a reduced concentration of about 0.09 μM. Very interesting is the GSH which, although very present in the blood cells and at the tissue level (mM range), in the plasma the reduced form of GSH is much lower (2-5.1 μM) (41). As for the HMW-SH, it should be considered that the plasma proteome is composed of about 3000 molecules; the concentration of thiol proteins is in the range of 400-600 μM and, as for the tissue PSHs, are targets of both reversible and irreversible oxidative modification. Human serum albumin (HSA) is the most abundant plasma thiol (43g/L) and is predominantly in its reduced form (75%, about 400 μM), about 2%, instead, has high forms of oxidation (sulphinic and sulphonic acids) and also having more than half of the protein mass in plasma (41). HSA is a monomeric protein with three domains; it consists of 585 amino acids (66438 Da) and 17 disulfide bridges (for a total of 35 Cys). Only one Cys does not participate in the formation of a disulfide bridge: the one in position 34 on the amino acid chain (Cys34). Cys34 residue is located in domain I with the sulfur atom oriented towards the inner part of the protein and, the binding of oxidizing species, other molecules (drugs), or the change of the conditions in which the protein is located

(pH for example), may lead to conformational changes in the protein. HSA is involved in several roles in plasma: one of the most important functions is to be responsible for the oncotic pressure, its domains have multiple points to bind and transport both endogenous and exogenous molecules, metals, vitamins, hormones, and drugs. HSA can be defined as a protein with antioxidant action also because of its ability to bond with metals: once bound to the domains of the protein, they are no longer bioavailable and can't participate in Fenton reactions, for example. What makes it a protein involved in the plasma redox network, however, is the presence of free thiol Cys34. In plasma, there is a mix of two types of HSA: mercaptalbumin presenting the thiol in position 34 reduced (HSA-SH) and non-mercaptalbumin having the thiol in oxidized form. In healthy young adults, the percentage of HSA-SH is much higher than in the oxidized form which, generally, consists of disulfides mixed with LMW-SH. Considering plasma as a more "pro-oxidant" compartment than tissues, the concentrations of LMW-SS and protein mixed disulfides are higher. Concentrations of CySS and disulfide for HCys (HCySS), CysGly and GSSG have levels ranging from, respectively, 41-63 μM, 1-1.2 μM, 4.4-6.8 μM and 0.7-1.6 μM (41). Considering that the Cys/CySS ratio is the major LMW-SH/LMW-SS system in the extracellular compartment, a high percentage of Cys will react with protein thiols to form S-cysteinylated proteins. Protein-linked Cys levels are in a range from 145-146 μM (about 65% of the total Cys in the plasma environment). About HCys, 70-80% of the total thiol form mixed disulfides with plasma proteins (albumin for the most), while 20-30% form homodimers or heterodimers with other thiols (42).

1.3-Plasma thiol pool and disease

Plasma is a good candidate for studying systemic oxidative stress due to its continuous interaction with tissues and the easy collection and separation during routine laboratory analyses. Moreover, plasma is the first compartment that counteracts xenobiotics even if the content of antioxidants is low. If we refer to thiols, LMW-SH concentration is very low, and they occur mainly in the oxidized form (LMW-SS and RSSP). The interest regarding the investigation of the redox forms of plasma thiols is because they can reflect systemic oxidative alterations and that (differently from the intracellular compartment where GSH/GSSG is generally investigated) it contains several redox thiol couples of potential clinical interests. The accurate measurement of the plasma thiol pool is not easy, due to the low levels of LMW-SH but, nowadays, there is general agreement regarding the physiological reference concentration in healthy people, and there is growing interest in the study of their alteration in pathological conditions. Imbalances in the ratio of the homeostasis thiol/disulfide may have a diagnostic and/or a pathogenic role so it could be useful to determine the oxidative framework of the whole body. This requires the measurement of the levels of free thiols and related disulfides comprising S-thiolated proteins. Additionally, the protein thiolation index (PTI) (43) is a new parameter that resumes the content of RSSP and the amount of free PSH present in the sample, thus making the evaluation of the plasma redox state highly accurate. PTI involves the measurement of the concentration of PSH that, taken alone, is not an indicator of oxidative stress because it can be influenced by the different concentrations of proteins. S-thiolated proteins, on the other hand, are much more present in plasma than in the tissue compartment (~30-70% instead of 1-2%); the difference is mainly due to the pro-oxidant characteristics of the extracellular compartment, in contrast to intracellular one. As mentioned above, the role of oxidation and the thiol level modifications have been investigated during different pathologies. One example is the Cys/CySS and GSH/GSSG ratios, which may vary in different conditions. Cys/CySS ratio imbalance to a more oxidized pattern is associated with oxidative phenomena of extracellular matrix substances. CySS is involved in fibroblast cellular proliferation and fibronectin production in pulmonary fibrosis (44). Aging, a physiological phenomenon, is a particular example where plasma thiol pools change with age. First, the concentration of HSA-SH compared to the oxidized form: if in younger individuals the amount of the reduced form is higher, in older individuals the oxidized form prevails.

Also, the concentrations of GSH and CysGly decrease with an increase, instead, of the oxidized forms; Cys and HCys, on the other hand, increase, and this is probably given by the outflow of these amino acids from various tissue districts (45). The balance between RONS and antioxidant mechanisms is the basis of the degree of oxidative stress to which the body is subjected during aging. Oxidizing species are involved in the accumulation of increasing levels of nuclear DNA damage in older organisms. In addition, mitochondrial DNA compared to nuclear, due to the proximity to the main source of production of cellular oxidizing agents (the mitochondria with cellular respiration processes) and the lack of an efficient DNA repair system, is much more susceptible to oxidative damage. An increase in mitochondrial DNA damage leads to an inevitable impairment of the functionality and integrity of the mitochondria themselves. It is hypothesized that the damaged mitochondria release a greater amount of ROS, thus triggering a vicious circle of increased damage to DNA that leads to greater production of oxidizing species modifying what is the redox balance of the organism towards a more oxidized pattern (12). In a study carried out by our laboratory (43), PTI also increases during aging. Plasma samples from 77 apparently healthy subjects aged between 21 and 87 were analyzed, and there was a near linear increase in PTI values with aging. These results confirm what is the "free radical theory" according to which aging is the result of cumulative damage induced by the production of endogenous radicals mostly of mitochondrial origin (12). Another study (45) of our laboratory evaluated the levels of plasma thiols of a group of 41 subjects between 21 and 92 years. Results suggest that there is a steady decrease in plasma levels of GSH and CysGly (tGSH and tCysGly remain unchanged) and an increase in tCys and tHCys over life. The increase in the concentrations of the latter is due to the increase in the disulfide forms of the two thiols. Changes to redox balance are related to increased production of RONS due to aging which tends to decrease all plasma thiols. As a response to this, the body tries to efflux thiols from tissues but, since tGSH and tCysGly remain unchanged, is assumed that there is no efflux of GSH and CysGly from this compartment. Cys efflux is balanced by the action of oxidizing species, the result is an increase in plasma tCys in which Cys remains unchanged but increases the oxidized forms (CySS and Cys protein mixed disulfides). Finally, the increase in plasma tHCys can be a consequence of the activation of TSP aimed at increasing the efflux of Cys for plasma. In addition to aging, a redox ratio of thiols/disulfides that tends towards a more oxidized pattern is also associated with other contexts: chemotherapy (46), chronic alcohol abuse (47),

cigarette smoking (48), and type 2 diabetes (49). Several are also the evidence that correlates cardiovascular diseases to an altered redox state: high concentrations of total Cys (reduced + oxidized + protein mixed disulfide) are related to a greater appearance of atherosclerosis and coronary heart disease (50, 51). In a study (52) involving 124 healthy subjects, a significant correlation was found between endothelial function and thiol oxidized species. Plasma levels of CySS and CySSP were important predictors of flow-mediated vasodilation, supporting the hypothesis that endothelial dysfunction is partly dependent on the overall extracellular oxidants/antioxidants balance. These parameters can therefore play a predictive role for future cardiovascular events. These results agree with another study (35) concerning inflammatory signaling in early atherosclerosis. Plasma LMW-SH disulfides, through thiol/disulfide exchange, oxidize thiol membrane proteins on the endothelial surface by starting a series of processes that increase the expression of cell-cell adhesion molecules and monocyte attachment to the endothelium. Elevated levels of γ-glutamyl transferase enzymatic activity in dyslipidemic subjects with metabolic syndrome are also correlated with a potential pro-oxidant plasma profile. The enzyme is more present in people affected by this pathology and is involved in the degradation of GSH in CysGly and Cys by removing the antioxidant effect of tripeptide. The profile is compatible with an oxidative stress framework and could be an emerging risk factor for cardiovascular disease (36). HCys, discussed in more detail in the next section, is another biomarker present in some pathological states. In summary, the alteration of the plasma redox balance accompanies the evolution of cardiovascular phenomena (although a pathogenetic pattern has not yet been described). Alteration of the thiol balance is also present in cancer: the role of thiols, in this case, is more complex because the variations are dependent on the type of tumor considered and the stage that the pathology has reached. Finally, in the course of neurodegenerative diseases, further studies are necessary as it has not yet been clearly described how the plasma pool of thiols reflects possible changes in the brain (53). The study (38) of 50 patients with Alzheimer's, however, showed higher plasma levels of tHCys and tCys than the physiological ones. Oxidative stress associated with the disease plays a key role in some processes of TSP and re-methylation cycle (figure TSP and re-methylation cycle). While methionine synthase of the re-methylation cycle is very vulnerable to oxidative stress, TSP cystathionine β-synthase is much more active in the oxidative environment. This is reflected in a decrease in methionine formation and a consequent increase in tHCys and tCys levels. The increased activity of TSP is probably

a physiological response to oxidative stress, one of the most important functions of this process is the synthesis of GSH (supplying the cell with Cys). Redox processes also play a fundamental role in the pathogenesis of atherosclerosis: their preponderant action is found during the cell signaling processes that manage the various phases of the cell cycle (proliferation, growth, senescence, and programmed cell death) which are associated with oxidative species production and thiol/disulfide exchanges (54). Inflammation itself is closely correlated with the alteration of the redox balance: the initial endothelial damage and the formation of the atheroma, seem to be triggered mostly by lipid oxidation (55). Plasma thiols pool, therefore, could substantially affect lipoprotein oxidation, the inflammatory response of white blood cells, and damage the endothelial membrane. A pivotal event in atherosclerosis pathogenesis is LDL oxidation resulting in macrophage uptake of lipoproteins, cell foam production, activation of pro-inflammatory responses, and endothelial dysfunction. Plasma redox state appears to be involved in the oxidation processes of plasma LDL (both directly and indirectly) although, physiologically, it acts in a way that inhibits LDL oxidation, thiols in fact can prevent or reduce the oxidation of LDL (56). However, some plasma components are also able to promote LDL oxidation: HCys, for example, is involved in the metal-dependent oxidation of lipoproteins (57). Furthermore, uncontrolled lipid oxidation can interfere with the production and maturation of HDL through various processes, removing the atheroprotective action carried out by the molecule. However, there are also more direct pathways that are not the consequence of the action of oxidized lipids: the action of neutrophil myeloperoxidase, for example, can oxidize cyanide to cyanate causing an accumulation of carbamylated substrates in the atheroma (58). Also, the presence of altered uric acid levels is associated with endothelial dysfunction (59) and carotid atherosclerosis (60), probably due to the pro-inflammatory action of urate crystals.

1.4-Hyperhomocysteinemia (HH)

1.4.1-Pathogenesis

Initially, rare inborn metabolic errors were described to lead to a condition called homocystinuria characterized by high levels of HCys in plasma and urine. Clinical manifestations of this condition are multiorgan and, patients suffering from this pathology, have a higher incidence of atherosclerosis and thrombotic disease (32). Inborn errors that lead to homocystinuria are borne by enzymes involved in the processes of transsulfuration or re-methylation of TSP: a defect in cystathionine-β-synthase (CBS) doesn't allow the HCys conversion into cystathionine causing an increase in HCys levels; instead, mutations in the enzymes methionine synthetase or methylenetetrahydrofolate reductase (MTHFR) inhibit the processes of re-methylation for the HCys conversion in methionine (32) (Figure 2).

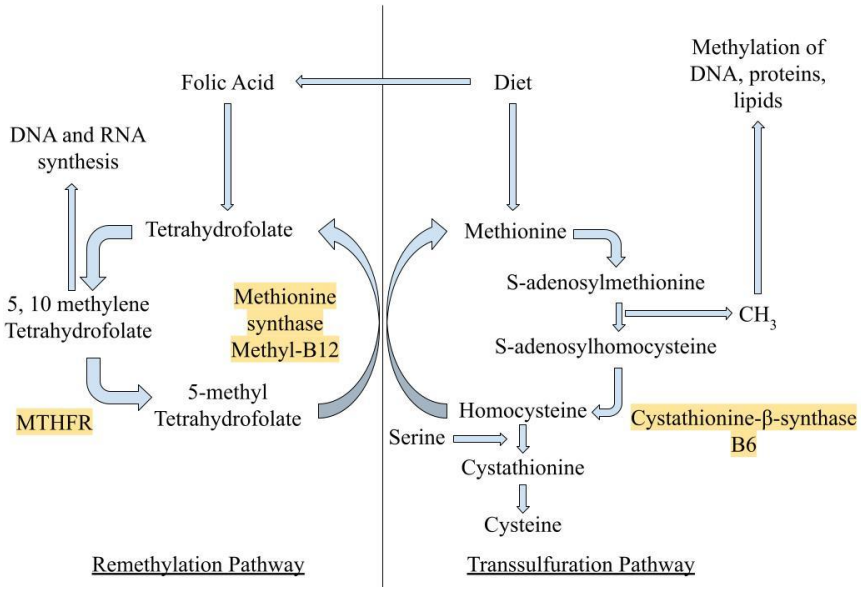


Figure 2. **Schematic representation of re-methylation and transsulfuration pathways**.

Several are the known and described reasons for which there are conditions in which HCys circulating levels are above the physiological levels. There is also a condition in which HCys levels show a slight increase compared to physiological values called mild hyperhomocysteinemia (mHH) with symptoms of vascular prevalence (61). Usually, normal values of serum HCys are in the 5-15 μmol/L range, the mHH is the condition in which levels of plasma HCys are between 12 and 30 μmol/L, moderate HH with values

between 31 and 100 μmol/L and severe HH when plasma concentrations of HCys are above 100 μmol/L (39) with a prevalence of 5-10%, 0.5-1%, and 0.01-0.03% respectively. HH may also be due to the lack of one to three basic nutrients that are co-factors in TSP and the re-methylation pathway: vitamins B9 (folate), B12 (cobalamin), and B6 (62). During the first step of TSP, CBS promotes the condensation of HCys and serine to form cystathionine, and vitamin B6, is necessary for the enzyme to perform its catalytic action. In the re-methylation cycle, however, HCys can be converted to methionine by obtaining a methyl group from vitamin B9 in a vitamin B12-dependent reaction (40). Not only that, in the liver and kidneys the lack of betaine (a by-product of choline degradation) does not allow the re-methylation of HCys to methionine by the enzyme betaine/HCys methyltransferase. Finally, the low intake and/or intestinal malabsorption of nutrients or pathologies of organs that deal with HCys metabolism can lead to conditions in which plasma HCys concentrations are significantly higher than physiological values. CBS-deficient homocystinuria is one of the most studied metabolic disorders of HCys; some studies, first, allowed the identification and later the association of CBS deficiency with homocystinuria (63) while later, other studies correlated high levels of HCys as a cause of vascular damage (64). Association between HCys and vascular phenomena was demonstrated because, patients with CBS deficiency, had high levels of HCys that were drastically lowered through the massive intake of vitamin B6 with a consequent reduction in the risk of vascular events; not only that, the simultaneous intake of other vitamins of group B (B9 and B12) allowed a further lowering of circulating levels of HCys. Patients not responsive to B6 treatment, however, were given a low methionine diet with free methionine and betaine fortification (65). These treatments, however, can reduce HCys plasma concentrations but never reach baseline values: post-treatment concentrations are higher than the levels found in the mHH pattern. It should be noted, for this reason, that even a small variation in HCys can be a risk factor or an amplifier for other risk factors in the development of vascular events. Over the last 20-30 years, numerous studies and meta-analyses have been conducted on HCys to verify if it is possible to consider it an independent risk factor for vascular phenomena when present in physiological values. Some studies support the hypothesis that the condition of mHH is considered a risk factor for cardiovascular events, especially atherothrombosis (66). However, there are also conflicting findings from prospective and observational studies that raise doubts about the possible causal role HCys has in the pathogenesis of cardiovascular disease (67, 68).

The correlation between cardiovascular disease and high levels of HCys has been investigated in a case-control study (69) where a significant increase in HCys was found in patients with coronopathies, compared to a control group, after a challenge with methionine. Later, a multicentric case-control study (70) revealed several information about the relationship between HCys and vascular disease both before and after a methionine load test. However, results obtained from these studies are contrasting with each other and helped to raise doubts about the real causal contribution that HCys has in vascular phenomena. Later, other meta-analyses described conflicting results: the first (71) shows that the increase in HCys is an independent risk factor for cardiovascular disease while, another more recent one, suggests that elevated HCys is at most a modest independent predictor of vascular phenomena in healthy populations (72). Prospective studies, moreover, although they overcome some limitations that can be found in case-control, present other problems mostly related to experimental design, end-points, and the genetic or nutritional differences of the populations studied. Some results can also be influenced by operator-dependent factors such as sample management in the picking, storage, and handling phases where the concentration of HCys, in case of bad management, can increase by up to 35%. This effect is generated by the almost constant rate of release of HCys by erythrocytes, these represent the major contributors to the artificial increase of HCys thanks to the combination of production and release in the extracellular compartment of the amino acid (73). Finally, the presence of concomitant risk factors, such as cigarette smoke or serum cholesterol, may also alter the results of analytical measurements (74). In this context, high serum levels of HCys may also be a consequence and not a cause of cardiovascular disease: it could be an unrelated side effect or a marker of a worse disease. A discovery that questions the cause-and-effect correlation between HCys and vascular phenomena involves a mutation in the MTHFR gene, specifically a thermolabile variant. Approximately 12% of the white population carries two copies of this variant leading to a slight increase in HCys levels. Despite this, it has been shown that having two copies of this variant does not necessarily increase the risk of cardiovascular disease, even having high levels of plasma HCys. In addition, the fact that patients with CBS deficits and HCys levels above normal, despite treatment with lowering agents, have small cardiovascular risk, again casts doubt on a direct cause-and-effect correlation (67, 75). Molecular pathways that accelerate atherosclerosis in people with HH are probably different from those involved in arterial wall damage of homocystinuria patients. While homocystinuria is a rare genetic

disorder characterized by extremely high levels of HCys due to specific enzyme defects, HH is a more common condition influenced by genetic factors, diet, and lifestyle in which HCys levels are high but not necessarily to such a severe extent. Further studies have shown that even a slight increase in plasma HCys (mHH) can adversely affect endothelial cells. In addressing the issue of the association between mHH and cardiovascular disease, the scientific community is engaged in a debate in which it is not yet entirely clear whether this correlation is solely due to its role as a diagnostic/prognostic factor or whether there is an actual underlying pathogenetic action. HCys has been the subject of numerous research studies, and among the mechanisms by which it is hypothesized to contribute to the pathogenesis of cardiovascular diseases, several approaches are distinguished. However, it should be emphasized that often these mechanisms are only hypothetical. Both the damage and dysfunction of the endothelium are induced by HH, high levels of the amino acid compromise vascular homeostasis and tissue perfusion. HH also increases platelet aggregation, and smooth muscle cell proliferation, and acts on the coagulation cascade and fibrinolysis by stimulating the conversion of normal endothelium into a pro-thrombotic pattern phenotype (76). Under certain conditions, HCys may exhibit pro-oxidant activity (77) and, most often, the harmful effects of HCys are pointed to oxidative stress damage for this reason. HCys in fact can undergo an auto-oxidation process by a transition metal-catalysed reaction at physiological pH and with oxygen availability. The results of these reactions are oxidizing species that, as previously seen, participated physiologically and/or pathologically in the thiols redox homeostasis. In addition, HCys can alter the function of certain enzymes involved in the buffering of oxidative damage such as GPx, the amino acid can decrease the action of the enzyme by modifying the translational mechanisms necessary for the synthesis of the selenocysteine-containing protein thus making tissues more exposed to the cytotoxic effects of oxidizing species (78). In addition, results of experiments on both human and animal models have shown that HH can impair vasodilation mediated by nitric oxide (NO) (79). The mechanism of action that elevated HCys plasma levels induce in inhibiting the vascular relaxation processes generated by NO involves an increase in the intracellular production of anion superoxide which, with NO availability, forms peroxynitrite depleting NO stocks and causing oxidative damage (80). HCys inhibits the production of NO also through the increase of asymmetric dimethylarginine (ADMA) in HH subjects; ADMA is an endogenous inhibitor of endothelial NO synthase that, with

high serum levels of HCys, is not catabolized to citrulline and methylamines (81). Other studies (82) have shown that HCys can stimulate the production of different types of pro-inflammatory cytokines involved in white cell recruitment thus favoring the development and progression of atherosclerosis. Also, another possible mechanism of vascular injury involves HCys creating stress to the endoplasmic reticulum by interfering with the formation of disulfide bonds and causing protein malformation and maturation in the organelle (83). Increased gene expression activated in response to endoplasmic reticulum stress results in a dysregulation of certain cellular functions involved in the development and progression of the atherosclerotic framework concerning lipid dysregulation, programmed cell death, and inflammation. It has also been observed that in all types of cells affected by atherosclerosis, there has been an increase in the production of lipids such as cholesterol and triglycerides (84). The role of HCys in subjects with HH also involves platelets that, with high plasma levels of the amino acid, are more activated and lead to an increase in thrombotic phenomena and proliferation of smooth muscle cells, pivotal phases of the atherosclerosis process (85).

1.4.2-Hyperhomocysteinemia therapy

As for therapeutic interventions, although the correlation between hyperhomocysteinemia, cardiovascular diseases, and molecular mechanisms of damage is not yet clear, the measures taken are aimed at reducing the incidence of cardiovascular phenomena by lowering the circulating levels of HCys with vitamin therapy or other strategies.

Vitamin B therapy

The use of folic acid, vitamin B12, and vitamin B6 refers to what is called “vitamin therapy”. The easy use of these natural and inexpensive cofactors offers an optimal approach to testing the lowering action against HCys. Studies carried out on animal models and small trials that included the measurement of end-point parameters such as endothelial function, oxidative biomarkers, and inflammation showed a positive finding in the use of vitamins as a treatment of HH (86). Other studies (87), instead, have tested the effectiveness of the therapy in lowering the HCys including secondary prevention parameters for cardiovascular and cerebrovascular diseases; the results showed mostly negative feedback although vitamins can decrease HCys levels. The contrasting results

between meta-analysis and experimental data can be given for different reasons: the measurement of highly reactive species such as HCys in the reduced form may be higher than the measurement of total HCys in the assessment for cardiovascular risk. HCys, as mentioned above, is present at the plasma level in various forms: in reduced form, such as symmetric disulfide (homocystine, HCySS) or asymmetric with other LMW-SH or mixed with proteins (88). HCys in reduced form is about 2-3% of the total, the remaining part is in the form of disulfide, mostly mixed with proteins; about 80% of the total HCys in the form of disulfide is mixed with proteins and only 20% is oxidized with other LMW-SH (mostly in the form of HCys-Cys) (45). Secondly, HCys may not be the determining factor in atherosclerotic disease as there may be other highly related metabolic species that are causing the disease. There is also the possibility that the biological effects resulting from the use of B vitamins may have more disadvantages than benefits. B vitamins increase the methylation potential (useful to re-methylate HCys to methionine). DNA methylation makes it possible to regulate gene expression, especially when it occurs in promoting regions and, in the case of atherosclerosis, plays a predominant role. Vitamin-B-therapy in the treatment of mild HH, therefore, can alter the methylation potential of the endothelium by promoting both a phenotype and pro-atherosclerotic pattern (86). The conclusion is that the use of B vitamins in the preventive treatment of mHH did not lead to the expected results; also, underestimating a highly complex metabolic pattern may have led to the conclusion that this type of treatment was effective. The debate is still open.

GSH enhancers

In this regard, research has begun to focus the study on compounds able to perform thiol/disulfide exchange: release protein-bonded HCys and promote urinary excretion with a consequent plasma level lowering (89). The HCys metabolic pathway consists of a re-methylation cycle present in all cells and tissues which contributes to the HCys extracellular pool, and TSP which is carried out mainly in the liver and kidneys (90). The study of new therapeutic interventions focuses on the latter part: increasing the uptake of HCys to the liver and kidneys to increase the excretion using compounds able to release protein mixed HCys or LMW-SH. HCys reduction to free thiol would facilitate cell uptake and, through TSP, its conversion to Cys; in the liver, it would become a substrate for the formation of GSH or, instead, degraded into taurine and sulfate (91). It should be noted that HCys urinary excretion is relatively low (only 2%

excreted with urine), HSA-linked HCys can't be ultra-filtered, and other HCys disulfides are highly resorbed in the urinary excretion process. Increasing GSH levels could offer several benefits in counteracting HH. As described above, GSH acts as a powerful antioxidant in the body, neutralizing oxidizing species and buffering oxidative stress associated with high levels of HCys. This action can help protect cells and tissues from the accumulation of oxidative damage. In addition, GSH can promote the detoxification of HCys when it's in the form of proteins mixed disulfide through thiol/disulfide exchange reactions, thus making the amino acid available for metabolism and/or excretion. Cardiovascular effects associated with HH can also be reduced by increasing GSH, which can be effective in improving endothelial functions, reducing inflammation of the blood vessels, and maintaining the structural integrity of the vessels. GSH enhancers can be categorized both into thiol and non-thiol compounds.

a) non-thiol compounds

Various nutrients (including vitamins and minerals) have also been studied by their enhancer ability for GSH. Vitamin B6 itself, as described above, being an enzymatic cofactor in TSP, performs its function by helping to convert HCys into Cys. Vitamins C and E have been studied for their antioxidant effects which could be mediated by the consequent increase of GSH (92); ascorbic acid, probably, allows a relative increase of GSH as it competes with the latter in reactions with RONS for electron donation. Ascorbic acid, however, also forms semidehydroascorbyl radicals that, being relatively non-reactive and easily reducible to ascorbate by a NADPH reductase, do not affect the reserves of GSH (93). Cruciferous vegetables have aroused great interest as they contain molecules that can increase GSH levels: sulforaphane and dithiolethiones. The subject of the study by the scientific community was a compound belonging to the second class: the anethole dithiolethione (ADT). ADT is a drug marketed in several countries both as choleretic and in xerostomia treatment, demonstrating good tolerability and safety when used at recommended therapeutic doses. In the late 80s' ADT (94) was shown to increase the GSH content in various organs, particularly the liver, both in humans and in laboratory animals by helping to protect against the toxic effect of acetaminophen. Later (95), its action was confirmed in our laboratory through *in vivo* studies in rats in which hepatic, renal, and encephalic GSH concentrations increased significantly after oral administration of ADT. In addition, the ability of ADT to improve the hyperhomocysteinemic condition in rats in which high blood levels of HCys were

induced via diet was evaluated. The results showed that there is a dose-dependent relationship in decreasing circulating tHCys levels. Although the mechanism of action is unclear, it is assumed that a decrease in the activity of the enzyme γ-glutamyl transferase by ADT would lead to a lower degradation of GSH as shown in another study (96).

b) cysteine pro-drugs

The most obvious method of increasing the concentration of cellular GSH is to administer it directly. The treatment *per os*, however, has poor bioavailability because, in the gut, the tripeptide is digested in its components, moreover, GSH is poorly taken at the tissue level. Although intranasal, intravenous, and sublingual administration also gave inefficient results (97), the use of GSH included in a liposomal carrier appears to be promising instead (98). To overcome the problems of cell permeability and bioavailability, the synthesis of modified GSH molecules began. GSH esters such as GSH ethyl ester (GSH-EE), after intraperitoneal administration, showed an increase in GSH levels in numerous tissues. In animal models, the administration of GSH esters *per os* showed an increase in GSH in kidneys and liver after pre-treatment with an inhibitor of GSH synthesis (buthionine sulfoximine) (99, 100) while, significant increases in GSH cell concentrations, were not found in *in vitro* experiments on HUVEC cells (101). GSH diesters were even more promising in being transported within cells resulting in increased levels of GSH and, although this mechanism is very rapid for intra/extracellular passage, inside the cell cleavage processes of the diester into monoester slows down its transport (102). The use of γ-GluCys has also been subjected to studies aimed at demonstrating its effectiveness as a way for increasing the GSH levels and, by providing this molecule, only the action of GSH synthetase is required to form GSH (103). Also, Cys can be considered an optimal strategy for the increase of the levels of GSH. The amino acid is considered the limiting factor in the synthesis of the antioxidant tripeptide; therefore, several pro-Cys drugs have been developed because Cys physical and chemical properties make it very unstable with a high tendency to auto-oxidize in the disulfide CySS which, among various features, there is its high insolubility. N-acetylcysteine (NAC, Figure 3) is a thiol compound that is largely used in clinical trials as a cysteine pro-drug (104). Once absorbed, it enters the cells where it is de-esterified and converted to cysteine. This should, then, increase GSH levels by promoting its enzymatic synthesis.

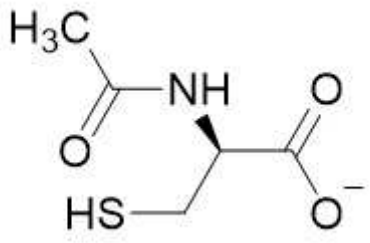


Figure 3. **N-acetylcysteine (NAC) chemical structure**.

It is well tolerated by the body and without adverse effects and is mainly used as an anti-mucolytic (especially in chronic obstructive pulmonary disease) and as an emergency drug acetaminophen poisoning. It has also been tested for its ability to perform thiol/disulfide exchanges and thus to try to reduce circulating levels of HCys. NAC administration in healthy subjects showed a change in the plasma redox/thiol disulfide balance with a dose-dependent effect already after 30 minutes with a recovery of the initial values after about 2-4 hours. Administration of NAC rapidly converted to Cys, likely displaces HCys and CysGly from protein binding sites via disulfide exchange reactions and leads to the formation of mixed LMW disulfides between NAC and Cys. These are easily eliminated by the kidneys and can probably become metabolically bioavailable for other liver-borne processes. As a result, the plasma levels of HCys and CysGly decrease (105). Results regarding the lowering of the levels of HCys through oral intake of NAC, however, are mixed (106, 107). One important limit related to the clinical use of NAC is due to its low hydrophobicity and bioavailability, thus making it harder to be able to reach therapeutic doses. Once inside the cells, NAC is de-acetylated into cysteine by the action of aminoacylases and several studies have tried to describe the kinetics of this reaction. *In vitro* (108), for example, it has been observed that oral administration does not significantly increase intracellular Cys levels due to both poor uptake of NAC by cells and slow de-acetylation. The cause may be an extracellular reaction with a Cys-containing disulfide to which NAC is subjected before entering the tissue (101). Although NAC appears to function as a GSH enhancer in animal and clinical trials (109), its poor bioavailability (about 10% after oral administration) (110) may influence the trial inconsistency results when repeated. Other *in vitro* and *in vivo* experiments indicate that NAC can increase cellular GSH levels (111, 112), but clinically significant results vary between studies. Oral treatment with NAC has shown success in restoring physiological levels of GSH in patients with HIV

(113, 114), autism, cystic fibrosis (115), and diabetes (116), however, its action may vary due to both the organ being examined and the pathological condition that caused organ damage. NAC treatment, moreover, may also be limited by the need to maintain high dosages of the drug for a long time, a condition quite difficult to implement. Other experiments suggest that NAC is unable to increase GSH levels in the central nervous system due to the difficulty it would encounter in crossing the blood-brain barrier (117). Finally, concomitant administration of NAC and glycine (GlyNAC) was proposed as a strategy to implement GSH concentration in elderly patients (118). Glycine and Cys are two of the three components of GSH whose concentrations, with aging, are lower compared to younger subjects. In a study carried out on elderly subjects, GlyNAC use showed a correction of the erythrocyte GSH depletion, also helping to reduce some signs of oxidative stress (119). Similar results, with a failure to reduce circulating levels of HCys over a long time, have been described with the use of dimercaptosuccinic acid (DMSA), a molecule very similar to the strong reducing compound dithiothreitol, having two free thiol groups per molecule (120). Finally, the use of sodium 2-mercaptoethanesulfonic acid (MESNA), an analogue of taurine, has also produced interesting but not entirely clear results; MESNA can exchange very quickly with HCys bound to albumin and increase the free fraction so that it can be processed and finally excreted at the renal level (89). It is yet to be clarified why some thiol compounds can decrease the levels of HCys in the short term, but with prolonged administration, they become less effective. This could be given, for example, to a greater efflux of HCys by cells because of treatments.

1.4.3-N-acetylcysteine ethyl ester (NACET)

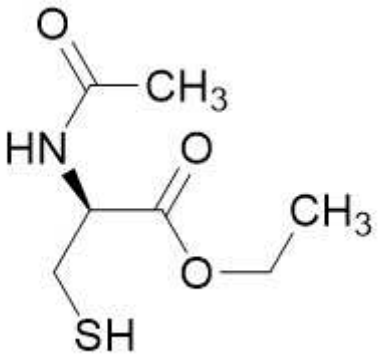


Figure 4. **N-acetylcysteine ethyl ester (NACET) chemical structure**.

A new, pro-Cys compound called N-Acetylcysteine ethyl ester (NACET, Figure 4) has been quite recently synthesized and studied to improve the pharmacokinetic properties of NAC. This molecule is characterized by a higher hydrophobicity than NAC (logD=0.85 for NACET versus -5.4 for NAC) (117) and demonstrates a better ability to pass through the cellular plasma membrane than NAC. NACET also has a bioavailability 10 times greater than NAC making it quickly absorbable from the gastrointestinal tract when it is taken *per os* and penetrating the tissues as a lipophilic substance. Intracellularly NACET is de-esterified to NAC which, being more hydrophilic, remains trapped inside the cell being slowly converted to Cys. Intravenous administration of NACET in rats showed that NACET can accumulate in various tissues as NAC and Cys and how, by administration of NAC and NACET in equivalent doses, only the latter can significantly increase the content of GSH in several rat organs and tissues (117). More specifically, chronic treatment in rats with equal doses of NAC or NACET showed a significant increase in tissue levels of GSH only with NACET. Of considerable importance, NACET has been able to increase GSH levels also in the brain which shows that the molecule can pass the blood-brain barrier. Another significant difference that emerged from the study is the ability of NACET to pass through the erythrocyte membrane faster, even if the molecule is not detectable within the erythrocytes. NAC, on the other hand, is the major metabolite of NACET that is being produced in human RBCs and given the lack of de-acetylation activity, tends to accumulate within RBCs. In rat erythrocytes, however, NACET is still able to enter and be converted to NAC but, here, it is also de-acetylated into Cys and exported. This evidence suggests that there is a higher de-acetylation activity in rat RBCs than in

human ones. *In vitro* experiments results on HUVEC cells where different enhancers of GSH have been tested, including NAC, GSH monoester, and 2-oxothiazolidine-4-carboxylic acid (OTC), showed that only NACET can significantly affect intracellular GSH (101). More specifically, NACET has been shown to have no dose-dependent action; concentrations higher than 0.5 mM gradually decrease intracellular GSH levels to concentrations similar to those of untreated cells. The hypothesis is that NAC, the NACET metabolite trapped inside cells, acts as a competitive inhibitor of GCS, given the similar structure that NAC possesses with Cys. Interestingly, both the GSH monoester and the OTC have been unable to raise GSH levels, even at higher concentrations, indicating results that contradict the literature. As for NAC, its action as a GSH enhancer results only when using high concentrations of the drug that are, however, outside the pharmacological range of use. NAC is not very effective at low concentrations due to its poor ability to permeate the cell membrane, low partition coefficient, and lack of a specific transporter. At high concentrations, however, only a small part can enter the cells, the extracellular amount then becomes available to reduce both CySS and Cys-albumin mixed disulfides to free Cys. The produced Cys can then pass inside the cell through specific transporters and increase the synthesis of GSH (101). The same GSH enhancing effect has also been demonstrated in other cell types (ARPE-19, retinal pigment epithelial cells) and, in the same study (121), *per os* administration of NAC and NACET in mice (50 mg/kg) showed a significant increase in ocular GSH levels only in subjects treated with NACET with a peak 4 hours after administration. Thanks to its promising pharmacokinetic properties, better than other pro-Cys drugs, NACET could be a valid alternative as a drug to increase GSH intracellular levels.

Aim

In the present study, we evaluated if and how NACET may be used to reduce circulating HCys levels in comparison with NAC. We evaluated the possibility that this decrease may be obtained by alteration of the thiol redox state in plasma. The study was characterized by a first step where a clinical study was performed enrolling healthy people. This study was carried out to evaluate the "thiolome" in these subjects and, therefore, the correlations between the different redox forms of thiols. Since NACET cannot be used for clinical evaluations, we then tested it in *in vitro* models, to evaluate the molecular mechanisms by which it may interfere with the metabolic pathways involving homocysteine.

Materials and Methods

2.1-Materials

All reagents were obtained from Sigma-Aldrich (Milan, Italy) unless otherwise indicated. Citrate buffer pH 4.3 was prepared by mixing 0.5 M sodium citrate with 0.5 M citric acid and stored at −20°C. The device used for *in vitro* experiments with oxidants was obtained from LabOmak (Siena, Italy).

2.2-Clinical study and blood collection

The study group comprised 62 consenting volunteers, recruited among co-workers, relatives, and friends. The group comprised 32 females and 30 males, ranging in age from 31 to 49 years old, none of them was an active smoker and all were abstemious or used to drink less than 20g of alcohol per week (the equivalent of a medium sized glass of wine or a pint of beer). All the participants reported that they were in good health, and none of them had any abnormality on physical examination or in routine laboratory blood and urine tests carried out from no more than 6 months (routine blood chemistry including lipid profile, blood glucose, complete blood counts, comprehensive metabolic panel, kidney, liver and heart functions, uric acid, electrolytes, iron and urine electrolytes, and glucose). All subjects were on a free diet. Consumption of N-acetylcysteine, vitamins C and E, and NSAIDs was avoided at least for the two months before analyses. Basal HCys levels were not considered an exclusion criterion.

Characteristics	Value
Age	40.3±6.5 (31-49)
Male weight (Kg)	75.6±5.1 (69-84)
Female weight (Kg)	68.8±6.6 (58-76)
Males height (cm)	174±7.0 (168-191)
Female height (cm)	169±10 (155-175)
Male BMI (Kg/m^2)	21.4±1.9 (19.3-24.0)
Female BMI (Kg/m^2)	21.1±2.8 (19.5-23.4)

Table 2. **Characteristics and values of the subjects considered for the study**. Average value plus SD for each characteristic and, in brackets, range of values.

Blood samples (3 mL) were taken from the antecubital vein in the morning after about 12 h of fasting, collected in evacuated plastic tubes containing K_3EDTA, and divided into different aliquots for analysis of thiols and disulfides in RBCs and plasma according to the procedure published by our group with some modifications (122). In detail:

2.2.1-LMW-SH in RBC

0.2 mL of blood was diluted three times with saline and centrifuged at 3,000g each time. Finally, the supernatant was accurately eliminated and 1 mL of 10 mM phosphate buffer pH 7.4 containing 1 mM mBrB (from stock 40 mM in MetOH) was added. Samples were stored at -80°C until analysis.

2.2.2-PSH and LMW-SH in plasma

500 µL of blood was stabilized with the immediate addition of a solution containing citrate buffer solution (see above). Plasma was obtained by centrifugation at 12,000g for 20 s carried out within 1 min after blood collection. 600 µL of plasma were diluted with 0.6 mL H_2O and 0.06 mL citrate solution and divided into aliquots of 0.1 mL each. PSH and LMW-SH content was immediately determined in one aliquot. The other aliquots were stored at −80°C until analysis.

2.2.3-Disulfides in RBC and plasma

800 µL of blood was transferred in tubes containing 80 µl of 310 mM NEM, after mixing by tilting the tubes ten times for one minute, the samples were centrifuged for 30 s at 8,000g. From the bottom of the tubes were then collected 0.3 mL of RBCs and, after 3 washings with 1 mL saline, the RBCs pellet was hemolyzed by the addition of 1 mL of 5 mM phosphate buffer, pH 6.5, containing 2 mM NEM and centrifuged at 20,000g for 15 min at 4 °C. Supernatant was collected and stored at -80°C for the analysis of both LMW-SS and Hb-SSP.

For the analyses of membrane RSSP, the pellets were resuspended with a glass rod in 5 mM phosphate buffer, pH 6.5, containing 1 mM NEM and centrifuged at 20,000g for 15 min at 4°C; this step was repeated three times and stored at -80°C until analysis (122).

2.2.4-PSSP in plasma and RBCs

These parameters were obtained by the measurement of total HMW-SS both in plasma and in blood cells and then subtracting the respective values of mixed disulfides with proteins. Five hundred µL of blood was immediately added with 80 µl of 310 mM NEM. After 5 min plasma was separated by centrifugation (14,000g for 10 s) and blood cells were washed three times with saline as described above. Samples were added with trichloroacetic acid (TCA, 3% (w/v) final concentration) and stored at -80°C until analysis.

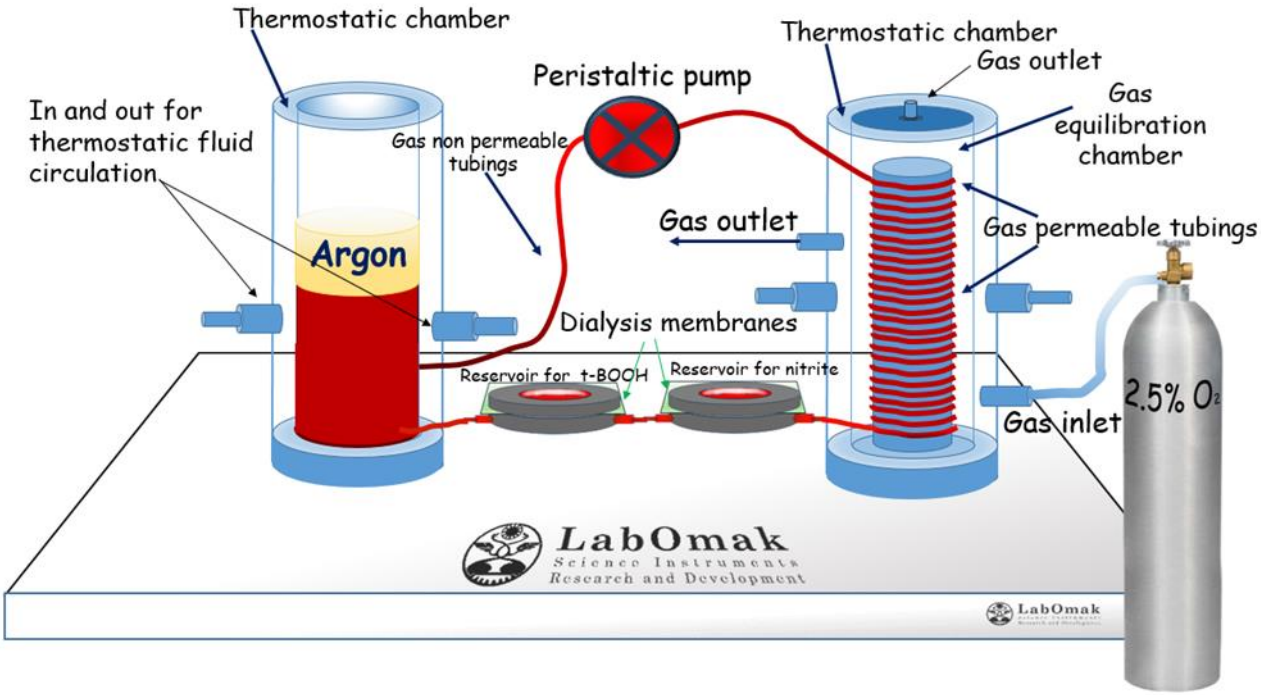
2.2.5-Other parameters of oxidative stress

The rest of the blood was used for plasma separation and analysis of the other oxidative stress parameters: protein carbonyls (PCO) and malondialdehyde (MDA). For these determinations aliquots of plasma (0.2 mL) for each parameter were stored at -80°C until analysis.

2.3-Blood collection for *in vitro* experiments with oxidants

10 ml of whole blood were obtained from the antecubital vein of 5 healthy donors involved in the clinical study. Blood was collected in evacuated plastic tubes containing K_3EDTA and used for in vitro experiments with oxidants. For the experiment, a specific device that allows us to perform the treatment under controlled conditions was used (Figure 5). More in detail, the device is constituted of two cylinders: one containing the blood and the other one a gas-permeable tubing (silicone tubing 1 m length, 2 mm outer diameter, 1.5 mm inner diameter, and 0.25 mm wall) from Silastic Medical Product (Dow Corning, MI, USA). Blood is fluxed throughout the two cylinders by a peristaltic pump. A gas mixture (2.5% O_2, 97.5% N_2) was delivered to the gas equilibration chamber. In the tubing that connects the two chambers are located two reservoirs that are constituted by two chambers: in the upper part can be located the oxidants, and the lower part is dedicated to blood flux. The two parts are separated by a dialysis membrane (10 kDa cut-off), with a contact surface of 4 cm^2. For this experiment, the peristaltic pump was set to a flux of 1.5 ml/min, and the temperature was maintained at 37°C through a thermostatic fluid circulation. A stock of glucose 1 M and glucose

oxidase 10 U/mL both dissolved in saline were added to blood to a final concentration of 10 mM and 0.1 U/mL respectively. This system is known to release hydrogen peroxide (123). Instead, tert-butyl hydroperoxide (t-BOOH) and sodium nitrite (0.5 mL of a 10 mM solution in saline each) were added to the reservoirs at 0, 30, 60 and 120 min, 2 mL aliquots of blood were collected and derivatized for analyses of RBC (GSSG, Hb-SSG and membrane S-glutathionylated proteins) and plasma (LMW-SS, RSSP and PTI) disulfides. Samples were stored at -80°C until analysis. Briefly: 0.8 mL of blood was derivatized as above for the disulfides analysis, 500 µL of derivatized (as above) blood for LMW-SH and PSH (PTI), and the remaining aliquots stored at –80°C for PCO



and MDA analysis.

Figure 5. Schematic representation of the device used for the *in vitro* experiments with oxidants.

2.4-Sample collection for the experiments carried out to evaluate GSH release from tissues and cells

2.4.1-GSH released from blood cells

Blood hemochromes were obtained from a local hospital, after routine analyses. Blood components were obtained by centrifugation under different conditions. Specifically: RBCs were purified by centrifugation of 0.5 mL blood at 10,000g for 30 s, removal of

supernatant, and three washings of pellets by saline. RBCs were then diluted by phosphate buffered saline pH 7.4 containing 5 mM glucose at a 25% hematocrit and used for the experiment. RBCs were incubated at 37° C, after 2 hours centrifuged at 10,000g for 30 s and finally, the supernatant was stored at -80°C until LMW-SH and LMW-SS analysis.

2.4.2-Polymorphonuclear and lymphocytes analysis

White cells were separated by PolymorphoprepTM (Fisher Scientific) starting from 5 mL blood (two aliquots of pooled hemochrome). Briefly, 5 mL of anti-coagulated whole blood was added to 5 mL of PolymorphoprepTM, after a room temperature centrifugation (500g for 30 min), two rings were obtained in the falcons, one for PMN and one for lymphocytes and monocytes. The cells were then collected and resuspended in PBS and glucose (as above), incubated at 37°C and collected after two hours. The calculations were normalized for mg/mL of proteins measured in cells.

2.4.3-Cell cultures

Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from umbilical cords collected from uncomplicated pregnancies and grown on 1% (w/v) gelatin (Sigma Aldrich) in EBM-2 medium (Lonza). A549 (human lung carcinoma-derived cells), IMR90 (human embryonic lung-derived fibroblasts), HEK293 (human embryonic kidney-derived cells), A375 (human melanoma-derived cells), HeLa cells were obtained from ATCCs (American Type Culture Collection Manassas, VA). All the cells were maintained in DMEM supplemented with 10% heat inactivated FBS, 100U penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. All cells were cultured in an atmosphere of 95% air and 5% CO2 at 37 °C in 100 mm culture plates. At the confluence culture medium was changed and after 24h collected and stored at -80°C for LMW-SH and LMW-SS analysis. Cells were collected and lysed for protein measurement.

2.5-Thiolome analysis

2.5.1-LMW-SH in RBC

Samples in mBrB after 10 minutes were deproteinized by treatment with 10 µL of 60% (w/v) TCA and centrifugation (2 min at 14,000g). LMW-SH were measured in the supernatant by HPLC.

2.5.2-PSH and LMW-SH in plasma

Protein thiols were measured by colorimetric reaction with Ellman's reagent (DTNB) (124) with some modifications. Briefly, 0.05 mL plasma samples, that had been treated with stabilizing solution (see above), were mixed with 0.8 mL of 200 mM Na_2HPO_4 and then spiked with 10 µL of 20 mM DTNB while continuously recording the colorimetric reaction on a spectrophotometer at 410 nm, until achievement of the absorbance plateau. LMW-SH were measured by deproteinizing plasma (0.1 mL) by treatment with 10 µL of 60% (w/v) TCA and centrifugation (2 min at 14,000g). Supernatant was then treated with 1 mM mBrB (final concentration), and the pH was brought to a slightly alkaline pH by addition of 20 µL 2M TRIS base. After incubation of 10 min in the dark, the samples were acidified by adding 5 µL of 33% (v/v) HCl. LMW-SH were measured in the supernatant by HPLC.

2.5.3-GSSG, RSSP, and mixed disulfides with proteins in RBCs

0.12 mL of hemolysed sample in NEM (see above) was deproteinated by adding 10 µL of 60% (w/v) TCA. After centrifugation (2 min at 14,000g), the supernatant was collected and used for the evaluation of LMW-SS. The extraction of the excess NEM was performed by treating the samples with two consecutive extractions of 10 min with dichloromethane on rotatory shacking. After centrifugation (2 min to 14,000g) the supernatant was recovered. Subsequently, 0.1 mL of extracted supernatant was treated with 2 µL of 5 mM DTT and 10 µL of 2 M TRIS base. After 20 min incubation at room temperature on rotatory shacking, 2.6 µL of 40 mM MBB were added and, after another 10 min incubation in the dark and acidification with 5 µL of 33% (v/v) HCl, LMW-SS were measured by HPLC (33).

RSSP were measured in the pellets that, before, were subjected to 1.5% (w/v) TCA washes: with a glass rod, the pellet was washed and centrifuged (2 min to 14,000g) twice. The pellet was then dissolved in 0.1 mL of 1 mM K_3EDTA by adding 2 µL of 5 mM DTT and basified with 10 µL of 2 M TRIS base. After 20 min incubation at room temperature, while stirring, 2.6 µL of 40 mM mBrB were added and the sample was subjected to further incubation in the dark for 10 min. Finally, after acidification with 5 µL of 33% (v/v) HCl, the measurement was made by HPLC.

The measure of membrane mixed disulfides with proteins was carried out as follows: 0,05 mL of membrane pellets were treated with 2 µL of 10 mM DTT after adding 10 µL of 2 M TRIS base. Following 20 min incubation at room temperature under stirring, 5 µL of 40 mM mBrB was added to the sample which was subjected to further incubation (10 min in the dark). After acidification with 5 µL of 33% (v/v) HCl, the analysis was performed by HPLC.

2.5.4-LMW-SS and RSSP in plasma

0.1 mL of NEM labelled plasma was diluted with 0.1 mL of 10% (w/v) TCA. After centrifugation (2 min at 14,000g), the supernatant was used for measuring LMW-SS and pellets for RSSP. LMW-SS: 0.1 mL of supernatant was treated with dichloromethane to remove excess NEM (see above). Subsequently, 0.1 mL of extracted supernatant was treated with 2 µL of 50 mM DTT and 20 µL of 2 M TRIS base and, after a first incubation at room temperature in stirring, the sample was treated with 8 µL of 40 mM mBrB and incubated in the dark for 10 min. Finally, the sample was acidified with 5 µL of 33% (v/v) HCl and analyzed by HPLC (33).

For the RSSP measurement, 0.1 mL of blood was treated with 0.1 mL of 4 mM NEM and left for 2 min in agitation. Samples were then acidified with 18 µL of 18% (w/v) TCA and, after a 2 min centrifugation (14,000g), the supernatant was discarded. Pellets were then washed with 1.5 % (w/v) TCA three times and, after the last centrifuge, resuspended in 400 µL of 1 mM K_3EDTA with 8.3 µL of 50 mM DTT and 15 µL 2 M TRIS. After 20 min in agitation, 100 µL of sample were acidified with 40 µL of 60% (w/v) TCA, and centrifuged (14,000g for 2 min). 50 µL of sample were then diluted in 150 µL of H_2O and then 100 µL were treated for 10 min in the dark with 3.2 µL of 40 mM mBrB and 20 µL of 2 M TRIS. Before HPLC analysis, samples were acidified with 5 µL of 33% (v/v) HCl.

2.5.5-PSSP in plasma and RBCs

Samples in TCA were centrifuged at 14,000g for 2 minutes. Pellets were resuspended with a rod glass in 1 mL of 1.5 % (w/v) TCA. After repeating this procedure four times, pellets were resuspended in 1 mL solution of 100 mM Tris-EDTA buffer at pH 8.5 containing 5 mM DTT. Samples were incubated for 30 min under rotatory shaking and successively proteins were precipitated by addition of TCA (5% w/v final concentration) and centrifugation (14,000g for 2 min). Pellets were washed with 1.5 % (w/v) TCA, resuspended, and precipitated again as described above until DTT disappeared from the supernatant (verified spectrophotometrically with DTNB, as above). Protein pellets were then resuspended in 100 mM Tris buffer pH 8.5 containing SDS under rotatory shaking. PSH were measured spectrophotometrically in these samples as follows: 10 mL samples were diluted in 1 mL of 100 mM phosphate buffer pH 8.0 and 10 µL of 20 mM DTNB. By the Lambert-Beer law, knowing the molar extinction coefficient of the DTNB, the thiol concentration of the test sample was obtained. This measure represents total HMW-SS, and PSSP were calculated by subtracting RSSP.

2.6-Analysis of PCO and MDA *in vivo* and *in vitro*

2.6.1-Protein carbonyls

Carbonylated proteins were derivatized with DNPH (123). Briefly, 200 µg (1 mg/mL) of whole protein cell lysates were mixed with 40 µL of 10 mM DNPH in 2 N HCl and incubated for 60 min in the dark. Samples were then mixed with 240 µL of 20% (w/v) TCA and incubated for 10 min in ice. After centrifugation at 20,000g for 15 min at 4 °C, protein pellets were washed three times with 1:1 ethanol/ethylacetate to remove free DNPH. Air-dried protein pellets were resuspended in 2× reducing Laemmli sample buffer. Proteins were separated by SDS-PAGE on 12% (w/v) Tris–HCl polyacrylamide gels, transferred to PVDF membrane and detected through Western immunoblotting with anti-DNP antibody (125, 126). Immunoreactive protein bands were visualized by ECL detection. Densitometric analysis of the bands allowed us to convert the measurement into numerical data and quantify the intensity of the light signal produced by each band.

2.6.2- MDA

MDA was determined by HPLC as previously described (127) with slight modifications. Briefly, 0.4 mL plasma was deproteinized by treatment with 40 µL of 60% (w/v) TCA. 0.2 mL of supernatants was reacted with the same volume of 0.6% (w/v) thiobarbituric acid (TBA) for 10 min in a boiling water bath in tightly capped glass tubes. Samples were then chilled in an ice-water bath and immediately analyzed by HPLC.

2.6.3-Total thiols (LMW-SH + LMW-SS) released from cells and tissues

0.1 mL of supernatant were treated with 5 µL of 10 mM DTT and 10 µL of 2 M TRIS. After 20 min incubation in stirring, 7 µL of 40 mM mBrB were added and, after 10 min of further incubation in the dark, samples were acidified with 5 µL of 33% (v/v) HCl and analyzed with HPLC.

2.7-HPLC separations

2.7.1-LMW-SH, LMW-SS, and RSSP (cytosolic and membrane) in RBC and plasma, LMW-SH and LMW-SS released from cells and tissues experiments

The chromatographic separation for the quantification of these biomarkers was carried out as follows: an HPLC Agilent 1100 series with a fluorometric detector (Agilent Technologies). For each set of analyses, the column was first conditioned with 100% mobile phase B (HPLC-grade acetonitrile), 1.2 mL/min for at least 5 min, and the temperature equilibrated at 25 °C. Then, the mobile phase composition was changed to 94% phase A (sodium acetate 0.25% (v/v) pH 3.10) and 6% phase B. Run conditions: 0–5′ 6% phase B, 5’-10’ gradient until 10% phase B, 10’-10′50’’ 10% phase B. The injection volume was 10 µL. After each injection, at the end of the run, the column was flushed with 100% phase B for 2 min, and then, the system was re-equilibrated to the initial isocratic conditions before the subsequent injection. The signals were recorded setting excitation at 390 nm and emission at 480 nm. Retention times: Cys = 4.18 min, CysGly = 5.27 min, HCys = 8.31 min, and GSH = 10.1 min. The retention factor for GSH is 4 (128). The column used was the HPLC Zorbax Eclipse XDB-C18 column 4.6 × 150 mm, 5 µm (Agilent Technologies).

Quantification was performed by using standard curves for individual analytes examined, dissolved, and titrated by spectrophotometry. The concentration ranges of the standards are 1-10 μM for Cys and 0.5-5 μM for the other thiols. The determination of the protein content, PSH values, and other thiols in samples was performed by a UV–Vis spectrophotometer (Jasco, V-750).

2.7.2-MDA

For each set of analyses, the column (same as before) was first conditioned with 100% mobile phase B (HPLC-grade acetonitrile), 1.2 mL/min for at least 5 min, and the temperature equilibrated at 25 °C. Then, the mobile phase composition was changed to 86% phase A (50 mM HEPES, pH 7.0) and 14% phase B (acetonitrile). Isocratic run conditions: 0–5′ 86% phase A. The injection volume was 10 μL. After each injection, at the end of the run, the column was flushed with 100% phase B for 2 min, and then, the system was re-equilibrated to the initial isocratic conditions before the subsequent injection. The signal was recorded setting wavelength at 532 nm. Calibration curves (50 nM to 1μM range) were built by adding known amounts of the MDA precursor 1,1,3,3-tetraethoxypropane to pooled supernatants.

2.8-NACET reactivity compared with other thiols

Equimolar solutions (1 mM, final working concentration of 20 μM) of MESNA (a chemotherapy adjuvant containing an -SH group), Cys, NAC, GSH, and NACET (synthetized by our group) were reacted with 0.2 mM DTNB in 0.2 M Na^+/K^+ phosphate buffer at pH 6.5 with an operating wavelength of 412 nm. Briefly, NACET was synthetized under argon atmosphere by N-acetylation of L-cysteine ethyl ester (Merck, Darmstadt, Germany) in dichloromethane with equimolar amounts of acetic anhydride (Merck, Darmstadt, Germany). HPLC analysis with UV (215 nm) absorbance detection of the isolated product revealed a chemical purity of >99% for NACET (117). Absorbance values of the Ellman's assay (124) were recorded and used for the speed constants calculations by fitting with a monoexponentially equation. Mathematical processing was done using the software Sigma Plot (Jandel Scientific San Raphael, CA, USA).

2.9-Cells treated with NAC and NACET

Cells were plated in a 96-well plate (1×10^4 cells per well). At the confluence, culture medium was changed and treatments with 0.2 mM (final concentrations) of NAC and NACET were performed. At 0, 4 8, 12, and 18 hours the medium was removed and stored at -80°C for total thiols analysis. Cells were washed twice with cold PBS and then lysed by adding 0.5 ml 4% (w/v) TCA containing 1 mM K_3EDTA. Samples were then collected and stored at -80°C for LMW-SH analysis. Stock solutions (20 mM) of the drugs (NAC and NACET) were prepared the day of the experiment in H_2O and then diluted to the final concentration in culture medium. In some experiments, HeLa cells were pre-treated with 0.5 mM probenecid (final concentration 200 mM stock dissolved in DMSO) for 1 hour before NACET treatment.

2.9.1-Total -SH in medium

0.1 mL of medium was treated with 0.5 mM of DTT (stock 10 mM) and 20 µL of 2 M TRIS. After 20 min stirring at room temperature, samples were deproteinized with 5% (w/v) TCA (from a 60% w/v stock). 0.1 mL of supernatant were then treated with 2 mM mBrB (stock 40 mM) and 20 µL of 2 M TRIS. After 10 min of incubation in the dark, samples were then acidified with 5 µL of 33% (v/v) HCl and analyzed by HPLC.

2.9.2-Intracellular LMW-SH

Supernatants obtained by centrifuging the samples stored at -80°C were treated with 7 µL of 40 mM mBrB and 20 µL of 2 M TRIS. After 10 min of incubation in the dark, samples were then acidified with 5 µL of 33% (v/v) HCl and analyzed by HPLC.

2.9.3-HPLC analysis

Elution conditions: solvent A = sodium acetate 0.25% (v/v), pH 3.09; solvent B = acetonitrile; 0–5 min: 94% solvent A/6% solvent B; 5–10 min linear gradient from 6% to 10% solvent B, 10–10.5 min linear gradient from 10% to 14% solvent B, 10.5–14.5 min 14% solvent B, 14.5–15 min linear gradient from 14% to 25% solvent B, 15–19 min linear gradient from 25% to 33% solvent B. A constant flow rate of 1.2 mL/min was applied. Detection was performed at 390 nm excitation and 480 nm emission

wavelength. In this HPLC system, NAC and NACET had a retention time of 13.9 and 18.6 min, respectively. A calibration curve for each thiol was constructed in the 1–500 mM range. If out of range, the sample was diluted with water and charged again. Standard solutions of Cys, γ-GluCys, GSH, NAC, and NACET were freshly prepared in water and titrated at the spectrophotometer by reaction with DTNB (124). The identity of the peaks was confirmed by pre-treating samples with N-ethylmaleimide or by addition of thiols.

2.10-Human plasma treatment with LMW-SH

Pool of the human plasma samples containing the highest HCySSP values (2.5 mL) was diluted 1:1 with PBS and placed at 37°C. The samples were then treated with 0.2 mM (20 mM stock) solutions of Cys, CysGly, GSH, NACET, and NAC; every hour for 8 hours 0.1 mL samples were taken and treated with NEM 10 mM (310 mM stock) for two minutes. After deproteinating the samples with 9 µL of 60% (w/v) TCA and after performing two consecutive washes with 1.5% (w/v) TCA to remove the unbound NEM, the reduction with DTT and derivatization with mBrB (as above) was carried out for determination of RSSP by HPLC as previously described.

2.11-Protein determination

Cell protein concentration was measured in acid precipitated pellets according to the Bradford assay (129) after dissolving them in 0.2 mL of 0.1 N NaOH.
Hb concentration was measured in an aliquot of hemolyzed RBCs by the cyanomethemoglobin method (130).

2.12-Statistics

Data are expressed as mean ± SD. Differences between means were evaluated by using ANOVA followed by Bonferroni posttest. A value of $P<0.05$ was considered statistically significant. Linear regression analyses were performed by applying the general equation: y=axþ+ b, where a is the slope and b is the intercept with the y-axis. Correlation analysis was performed by calculating Pearson's product-moment correlation coefficient.

Results and Discussion

Although HH is identified as a risk factor for cardiovascular disease, therapies that aim to reduce circulating HCys levels using vitamin B (vitamin B therapy) do not appear to significantly improve prognosis. This finding raises questions about the exact role of HCys in the pathological process of cardiovascular diseases and underlines the complexity of its metabolism. Since the level of HCys is affected by exchanges of thiol/disulfide with other thiol compounds in the body, an analysis of the "thiolome" has been conducted in blood samples from a group of healthy subjects, to better understand the underlying metabolic dynamics and possible implications for the prevention and treatment of cardiovascular disease course. This targeted approach provides a more comprehensive perspective on the relationship between HCys and cardiovascular health and may detect new therapeutic or risk management strategies.

3.1-Blood thiolome and parameters of oxidative stress in healthy people

The results of thiolome analysis and oxidative stress parameters in healthy subjects are shown in Table 3. The reported data include measurements performed for the evaluation of LMW-SH (GSH, Cys, CysGly, HCys, γ-GluCys), LMW-SS (GSSG, CySS, CysGlySS, HCySS, γ-GluCySS), HMW-SH (PSH) and HMW-SS (PSSP) of major physiological interest. In addition, the values of the thiol/disulfide ratios have been reported, which, as described above in some physiological processes and pathological conditions, undergo variations. Since most of the plasma GSH is contained at the RBC level, Hb and erythrocyte membrane proteins S-glutathionylation have been analyzed as well as measuring the RBC membrane protein S-thiolation with other LMW-SH. Finally, plasma concentrations of LMW-SH/proteins mixed disulfides of higher biological interest (CySSP, CysGlySSP, HCySSP, GluCySSP, GSSP) were analyzed along the total sum of these mixed disulfides (RSSP) and the PTI. Table 3 has also been divided into intracellular (RBC) and extracellular compartments.

Parameter	Intracellular		Extracellular	
	Range	Mean±SD	Range	Mean±SD
GSH	7.85-8.56	8.21±0.058 nmol/mg Hb	0.922-5.06	2.64±0.52 µM
GSSG	9.35-17.7	12.3±0.1 µmol/mg Hb	0.536-2.25	1.11±0.33 µM
GSH/GSSG	759-888	821±168	1.01-2.97	1.88±0.52
Cys	0.064-1.02	0.078±0.0033 nmol/mg Hb	7.6-18.8	12.3±0.560 µM
CySS		NA	43.2-64.0	59.8±8.40 µM
Cys/CySS	-	-	0.079-0.152	0.11±0.06
CysGly		NA	0.96-4.58	3.44±0.09 µM
CysGlySS		NA	4.62-6.66	5.60±0.21 µM
CysGly/CysGlySS	-	-	0.31-0.48	0.41±0.01
HCys	0.017-0.036	0.029±0.0011 nmol/mg Hb	0.133-0.256	0.183±0.061 µM
HCySS		NA	1.86-3.55	2.12±0.88 µM
HCys/HCySS	-	-	0.024-0.069	0.042±0.007
γ-GluCys		0.046 ± 0.0023 nmol/mg Hb	0-1.22	0.821±0.102 µM
γ-GluCySS		NA		NA
γ-GluCys/ γ-GluCySS		NA		NA
LMW-SH/LMW-SS	759-888	821±166	0.147-0.48	0.302±0.031
PSH	14.5-20.6	16.4±2.61 nmol/mg Hb	321-516	424±83 µM
PSSP	0.162-0.284	0.235±0.011 nmol/mg Hb	22600-34830	27000±5430 µM
HbSSG	7.25-12.6	9.14±1.13 µmol/mg Hb		NA
MP-SG	11.2-19.8	15.0±2.55 µmol/mg Hb		NA
MP-SX	12.6-22.4	19.5±4.0 µmol/mg Hb		NA
CySSP plasma		NA	102-148	125±29 µM
CysGlySSP plasma		NA	7.2-13.4	11.2±2.1 µM
HCySSP plasma		NA	4.23-16.7	9.36±2.39 µM
γ-GluCySSP plasma		NA	0.126-0.584	0.330±0.024 µM
GSSP plasma		NA	0.954-3.68	1.84±0.662 µM
RSSP plasma		NA	116-155	140±22 µM
PTI		NA	0.261-0.688	0.486±0.027
SHtot/SStot	522-843	653±114	$2.9x10^{-5}$-$5.1x10^{-5}$	$3.7x10^{-5}$

Table 3. **Intracellular and extracellular thiolome values in blood of healthy subjects (n=62)**. Data are reported as the mean±SD. The lowest and the highest values for each parameter are also indicated. NA: not available.

The first thing that can be noticed is that there is a substantial difference between thiols and disulfides in the two compartments. At the intracellular level, in a predominantly reducing environment, thiols are present at millimolar concentrations and, mostly, in their reduced forms. At the extracellular level, predominantly more oxidizing environment, LMW-SH are present in μM concentrations and LMW-SS or RSSP are the most present redox form.

Within cells, GSH occurs at the highest concentration (8.21±0.058 nmol/mg Hb) compared to the other LMW-SH that are, instead, present at micromolar concentration (mainly in reduced form). The values of the other LMW-SH considered in our experiments such as Cys, HCys, and γ-GluCys, have very low values at the intracellular level, respectively 0.078±0.0033 nmol/mg Hb, 0.029±0.0011 nmol/mg Hb, and 0.046±0.0023 nmol/mg Hb, or even below the detection limit (as for CysGly levels). In any case, HMW-SH are even higher than GSH, with Hb representing the most abundant thiol. In human blood, we can find relatively high concentrations of Hb (8-10 mM, as a monomer). The human Hb is characterized by the presence of only a couple of thiols per tetramer in the Cysβ93 position; its reactivity, moreover, is also defined by the quaternary conformation in which the Hb is found with, for example, a lower reactivity when it's in the deoxy state. Interestingly, rat Hb is different from human Hb: it has additional Cys in Cysβ125 and Cysα13 positions. Cysβ125 has been shown to have a lower pKa, better accessibility, and, consequently, greater reactivity. Later kinetic studies have confirmed that rat Hb is much more reactive than human Hb (131) and, these results, showed that rat blood is not the best choice for certain types of biochemical/physiological analysis. Oxidized forms are quite low within cells, both as symmetric disulfides and mixed disulfides with proteins. For most parameters, the values were under the detection limit. The oxidized forms of GSH were the most abundant with values of about 12.3±0.1 μmol/mg Hb for GSSG and 24.1±3.7 μmol/mg Hb for S-glutathionylated proteins (Hb and membrane proteins), GSSG is the most present disulfide and the GSH/GSSG ratio is the parameter that can be used to evaluate the redox potential in the cell, with values >800 intracellularly compared with the extracellular ones (values <2) according to the results of our experiments. As mentioned above, low values of this ratio (and therefore higher concentrations of GSSG than GSH) are indicative of pathology and certain physiological processes (such as cell proliferation or apoptosis, for example) (132). The values of intracellular GSH measured by our research group are similar to those of other studies with healthy

subjects with an average age that does not differ too much from the average of the subjects examined in our experiments. In a study that correlates low levels of GSH and high levels of cysteinylated and glutathionylated Hb in haemodialyzed patients against healthy subjects (133), blood samples from 21 healthy subjects with an average age of 56.7 years were analyzed. The mean value of RBC GSH measured was 8.46±1.75 nmol/mg Hb which, despite the greater age of the subjects analyzed, does not differ too much from the values obtained by our group (8.21±0.058 nmol/mg Hb). Interestingly, haemodialyzed subjects also have a very similar average value (8.72±2.5 nmol/mg Hb). In another study to assess plasma GSH levels in patients with Friedreich's ataxia (134), tripeptide values were also analyzed in 20 healthy subjects aged 8-22. The average value of what was described in the study as free GSH (GSH+GSSG) of healthy subjects was about 8.4±1.79 nmol/mg Hb which, although it also includes GSSG levels, shows little variance compared to our findings. Also, in another study to establish the role of exercise-induced oxidative stress in erythrocytes at different stages of their life cycle (young, middle-aged, old) (135), GSH levels in RBC were evaluated. The study analyzed the blood of 8 healthy subjects with an average age of 21±3 years and the results showed that, in older RBCs, the concentration of GSH was higher than in the younger ones with an average value of 8.4±0.4 nmol/mg Hb. Finally, our research group found similar values for RBC GSH (8.48±0.69 nmol/mg Hb) in a paper aimed at describing the conditions and the preparation of body fluid samples for a correct HPLC analysis (122).

As for the extracellular compartment, a strongly oxidizing environment, it can be inferred from Table 3 that the most present LMW-SH is the Cys and, therefore, the Cys/CySS ratio is the most indicative parameter for redox variations of the plasma environment and, despite being the most present LMW-SH, Cys concentrations are relatively low (mean value: 12.3±0.560 μM). On the outer surface of the cellular plasma membrane there is also a series of enzymes that deal with extracellular degradation of GSH. γ-GT metabolizes GSH in CysGly and glutamate and the extracellular CysGly concentrations in our study, compared to those of other LMW-SH, are worthy of mention (3.44±0.09 μM) and in line with other studies performed on healthy subjects (41, 136, 137). The subsequent action of an extracellular dipeptidase converts CysGly into the other two basic components of GSH (Cys and glycine) and, as already mentioned, plasma Cys concentrations are the highest among LMW-SH. Another metabolite of GSH is, of course, the oxidized form GSSG which, due to the pro-

oxidizing plasma environment, is at higher concentrations than the intracellular environment both in our results (1.11±0.33 μM) and in other studies (41, 136, 137). The most present redox forms of the thiols that we analyzed are the oxidized ones, both to form symmetrical disulfides and protein mixed disulfides (Figure 6). For example, CySS has an average value of 59.8±8.4 μM, significantly higher than the plasma Cys value (12.3±0.56 μM). LMW-SS are present in larger quantities compared to the LMW-SH concentrations as are disulfides between LMW-SS and proteins. While at the intracellular level proteins are mostly S-glutathionylated, in plasma Cys is the LMW-SH that is most involved in forming S-cysteinylated proteins. About HMW-SH, it can be noted that PSHs are the most present thiols in plasma where, HSA, represents the thiol with higher concentrations. Our results showed an average plasma PSH concentration of 424±83 μM, a value in line with other studies carried out on blood samples of healthy adults (41, 137). Plasma protein thiols that we measured in the group of healthy subjects have a concentration ranging from 321 to 516 μM and, as mentioned above, by the presence of the cysteinyl residue in position 34 of the amino acid chain (Cys34), HSA is the most abundant protein thiol (41). Cys34 may be present in several redox forms, in younger subjects generally 70-80% of HSA is present with Cys34 containing the free sulfhydryl group (HSA-SH, mercaptalbumin). Another 20-30% of HSA has Cys34 forming disulfides mixed with LMW-SH (non-mercaptalbumin): the most involved thiol is Cys and the concentration of plasma CySSP measured by us is much higher (125±29 μM) than other LMW-SH mixed with proteins (137). A small fraction of Cys34 (about 2-5%) is instead highly oxidized to form sulfonic and sulfinic acids (138). With aging, the percentages of these redox forms of Cys34 tend to switch towards a profile with higher concentrations of non-mercaptalbumin (137).

Considering the intracellular levels of HCys it is possible to see from Table 3 that these are relatively low at the limit of quantification. At the plasma level, however, the concentrations are more relevant, if we consider tHCys (HCys+2xHCySS+HCySSP) the average value obtained (Table 3) is in the physiological range (mean value 13.9±0.9, range 6.13-19.4) (39). Moreover, it can be shown from Figure 6 that HCys, compared to other aminothiols present in the plasma, is the LMW-SH that participates more in the formation of disulfides and, in particular, bound to proteins to form mixed disulfides (HCySSP). HCys is normally considered as tHCys, as above, but generally exists in three different redox forms that can have different metabolism and participate in different redox reactions (88).

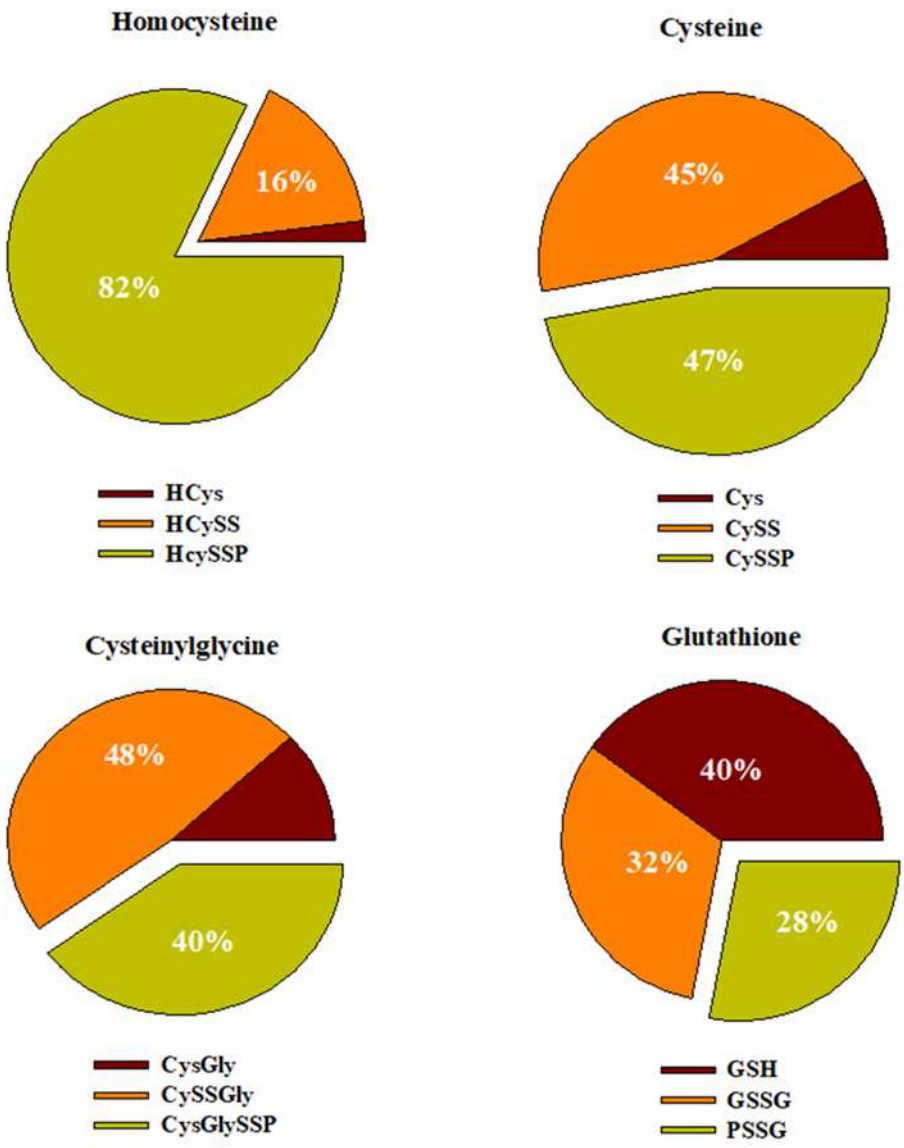


Figure 6. Schematic representation of the major redox forms for HCys, Cys, CysGly, and GSH measured in the plasma of healthy people. The percentage of each form was calculated considering the mean value reported in Table 3).

HCys mixed disulfides are generated by reactions of thiol/disulfide exchange between HCys and cysteinylated albumin. In a study about the roles of albumin and ceruloplasmin in the formation of HCys and Cys disulfides (139), it was found that HCys is the most involved thiol species in HCySSP formation. The study showed that copper or ceruloplasmin-catalyzed autooxidation of HCys is probably a minor process in plasma compared to the other aminothiols examined. The differences between the various copper-mediated oxidation rates of LMW-SH may be due to differences between the pKa of the -SH groups of thiols. The pKa of the sulfhydryl group of HCys

is at least an order of magnitude greater than Cys and CysGly, this allows to have, therefore, a lower level of the anion of HCys in the plasma compared to the other two LMW-SH, limiting the reactivity of HCys with copper. In addition, albumin is responsible for converting HCys into its oxidized forms either through the formation of HCySS or HCySSP in a dose-dependent manner. The formation of CySS by the catalytic activity of ceruloplasmin allows the formation of a mixed disulfide by thiol/disulfide exchange between albumin (in Cys34) and CySS. At this point, HCys can react with cysteinylated albumin to form homocysteinylated albumin with Cys release.

Considering the peculiar abundance of the oxidized form of HCys in human plasma, in comparison with the other thiols, we wanted to investigate the possible reasons for this property. Therefore, first, we evaluated if this unbalance toward the oxidized forms of HCys was possibly due to the occurrence of oxidative stress in our patients. In addition to the different redox forms of blood thiols, we also measured additional biomarkers of oxidative stress in the blood samples of the people enrolled in our study. Oxidative stress is defined as the inadequate inactivation of RONS by the body's antioxidant defenses. The process takes place either through a higher production of oxidizing species or through a loss of endogenous antioxidants (or even through both phenomena). The major consequences of oxidative processes are mostly borne by biomolecules of physiological interest such as nucleic acids, lipids, and proteins that can lead to impaired cell viability and induce different phenomena of cellular response by producing a chain of events that leads the tissue to necrosis or apoptosis. Not only, in the intracellular compartment, which is mainly being reduced (as denoted by the concentrations of thiols in Table 3), it is a proliferative stimulus for the tissue; a slight shift towards a weakly oxidized environment is associated with cell proliferation. It is now established, however, that oxidative stress and the production of RONS, are closely involved with aging and pathological processes (HH, for example) (132). The evaluation of oxidative stress is a very complex operation and the definition of biomarkers that can objectively measure and evaluate the redox state of an individual is far from simple. Although some RONS are detectable *in vitro*, their quantification is more difficult if applied for clinical examination due to the increased instability of different reactive species and/or equipment costs. Furthermore, RONS are generally very reactive and have a very short half-life which makes it even more difficult to measure directly in cells/tissues and/or body fluids. It is easier, therefore, to evaluate the effects that oxidized species generate by analyzing the redox state of some parameters

or the production of secondary products/modifications to oxidative targets (lipid peroxidation and protein oxidation, for example). However, the products of oxidative phenomena may differ depending on the type of oxidizing species prevalent. It's important to note that no single biomarker can provide a concrete assessment of an organism's oxidative stress. So, it's necessary to integrate different biomarkers to get a general picture and fully understand the complexity of this phenomenon. Starting from the evaluation of the alterations of the redox state of an organism following oxidative phenomena, some of the most used parameters involve the evaluation of the ratios between thiols and their oxidized forms (disulfides) as well as the degree of protein thiolation. The most important thiol/disulfide ratios include GSH/GSSG and Cys/CySS. The intracellular redox homeostasis is determined mostly by the ratio GSH/ GSSG. Maintenance of a physiological value of this parameter is vitally important both for cell survival and to regulate the redox state of protein thiols affecting their activity and function. Variations in this ratio can cause oxidative phenomena and/or reactions of thiol/disulfide exchange to the cysteinyl residues of proteins in the thiol proteins. A lowering of the GSH/GSSG ratio, under oxidizing phenomena, is given by both the decrease of GSH and the increase of GSSG. The redox balance, however, can be buffered by the enzymatic reduction of disulfide or the outflow of GSSG from cells helping to preserve cell viability. The GSSG is not taken up by the cells but is degraded at the extracellular level thus granting the *de novo* synthesis of GSH by cells. The increase of the levels of GSSG, moreover, permits protein S-glutathionylation that, as mentioned above, is a physiological mechanism that allows, through reactions of thiol/disulfide exchange, to protect the cysteinyl residues of proteins from irreversible changes due to oxidative stress, as well as to encourage the storage of GSH (37). In addition to GSH/GSSG we have developed and proposed a new biomarker of oxidative stress, named PTI, the molar ratio between protein mixed disulfides and free protein thiols in plasma. PTI is a parameter that works well on samples of human origin, when applied to samples of animal origin, especially rodents, the measure becomes more difficult to implement. Rats and mice have different thiol/disulfide plasma pools; for example, Cys34 albumin in rodents is much more reactive as well as much higher Cys plasma concentrations (even between different strains of the same species) (33, 140). The advantages of using PTI as an oxidative stress biomarker lie in the fact that thiol residues (-SH) are the most reactive groups in proteins and, in addition, thiol proteins circulate in the plasma for days before being removed from the bloodstream by acting as

"circulating thiol sensors" (43). PTI was measured in our samples (Table 3) but the values we obtained were in the physiological range, according to our previous data (141).

About the evaluation of changes to targets sensitive to oxidative stress, the analysis of lipid peroxidation products can complement the overall picture of damage from oxidizing species. The peroxidation of membrane lipids can be very harmful as it leads to an alteration of the biological properties of the membrane itself (loss of the degree of fluidity, inactivation of membrane complexes, alteration of permeability). The lipid oxidation process generates a series of particularly stable end products, mostly aldehydes, which can be measured in biological samples as an indirect index of oxidative damage (132). In this work, malondialdehyde (MDA), a physiological ketoaldehyde produced by the peroxidative decomposition of unsaturated lipids as a by-product of arachidonate metabolism, was considered. In addition to lipid peroxidation, other oxidative modifications can be investigated as biomarkers of oxidative stress, such as DNA modifications. Cellular DNA can be damaged by ROS under different conditions and several techniques have been developed to assess the damage induced to DNA nucleobases. Also, the non-enzymatic peroxidation catalyzed by free radicals of the esterified arachidonic acid leads to the formation of isoprostanes, a series of compounds widely studied as biomarkers of oxidative stress (132).

We selected carbonylated proteins (PCO), as an additional index of oxidative damage to be investigated. PCOs are generated by the oxidation of different amino acids on side chains and are used to evaluate severe degrees of protein oxidation both *in vitro* and *in vivo*. The content of PCO is the most general parameter and is commonly used as a marker of protein oxidation, the increase in PCO has been observed in several human pathologies and, generally, the evaluation of these values does not require special or expensive equipment (142). PCO analysis has several advantages over lipid peroxidation products as indicators of oxidative stress; generally, oxidized proteins are more stable, and PCOs form early and circulate in the bloodstream for much longer periods than parameters such as GSSG and MDA. PCO formation is also a common phenomenon during oxidation, and quantification of these modifications can be used as a measure of the severity of oxidative damage (143). Plasma thiolomics reported in Table 3 as well as biomarkers of oxidative stress were correlated to verify if an increase of tHCys can derive from an oxidant unbalance or just from pro-oxidant conditions (Table 4, Appendix).

We must remember in this context that the extracellular compartment is essentially void of antioxidants and even a minimal increase in oxidative stress might decrease circulating thiols and generate more disulfides. An increase in oxidative stress can shift the balance and lead to the formation of poorly eliminable HSA-HCys mixed disulfides. In a few words, oxidative stress may be the culprit of HCys. However, no correlation was found between biomarkers of oxidative stress and HCys. Moreover, no correlation was present between biomarkers of oxidative stress and thiols, disulfides, and thiol to disulfide ratios in the plasma milieu too, suggesting that the thiol to disulfide balance of plasma is minimally influenced by oxidative stress and probably regulated by other factors.

To have confirmation about the reliability of the parameters we used to detect oxidative stress in the clinical study, we measured the same biomarkers in an *in vitro* model. Specifically, we treated human blood with a mix of oxidants under controlled conditions in terms of flux and oxygen saturation of blood. For this aim we used a new device, set up for this purpose (Figure 5). This device allowed us to deliver the oxidants at a low flux and to maintain blood in a partially oxygenated condition (60% saturation Hb) through a dialysis membrane, this both simulates what happens *in vivo* (oxidants slowly delivered from surrounding tissues) and reproduced blood oxygenation where is maximal superoxide production from partially oxygenated hemoglobin (144). To our knowledge, this kind of approach is quite new for in vitro protocols of investigation on oxidative stress. The results of this study are reported in Figure 7.

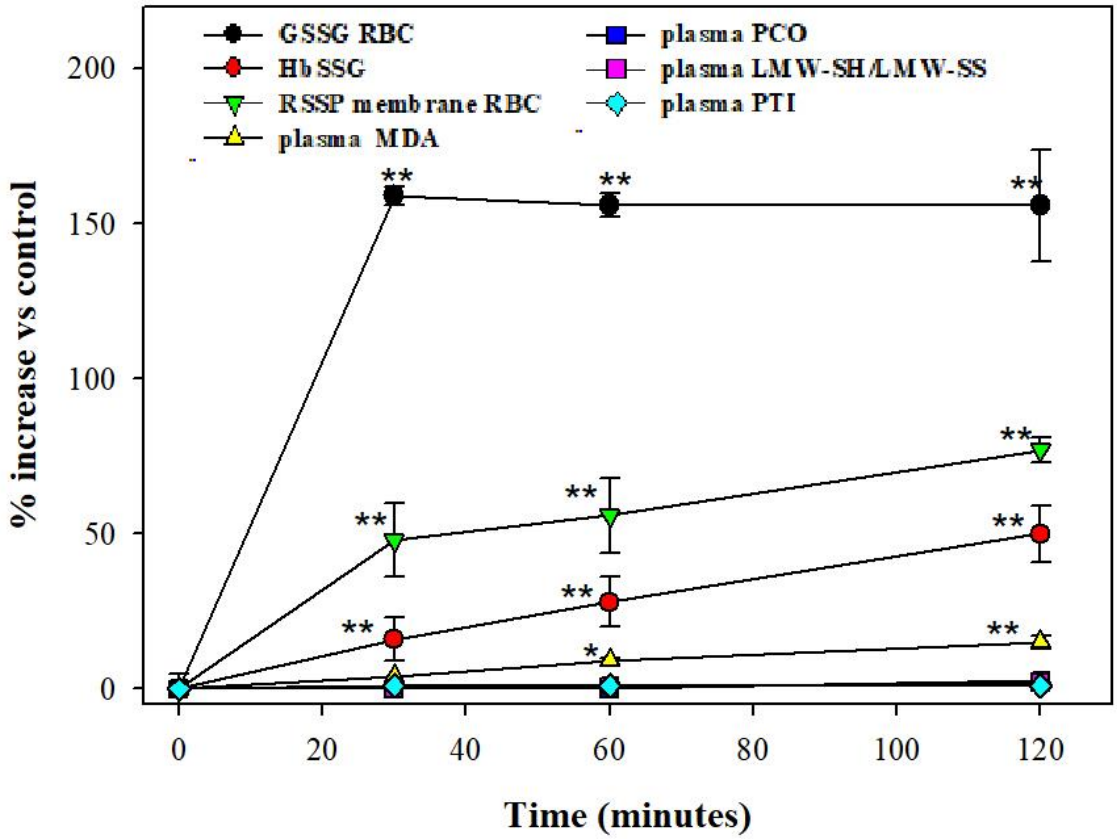


Figure 7. **I*n vitro* treatment of human blood with a mixture of oxidants (Glucose/Glucose oxidase, t-BOOH, and sodium nitrite)**. Oxidants were delivered at a low flux (1.5 ml/min) 37°C. Analyses of several biomarkers of oxidative stress both in plasma and RBCs were carried out at 0, 30, 60, and 120 minutes. GSSG = glutathione disulphide, RBC = red blood cell, HbSSG = S-glutathionylated hemoglobin, RSSP = protein mixed disulfide, MDA = malondialdehyde, PCO = protein carbonyls, LMW-SH = low molecular weight thiol, LMW-SS = low molecular weight disulfide, PTI = protein thiolation index. Data are reported as the mean±SD. **p<0.01 vs 0 time; *p<0.05 vs 0 time.

The parameters measured are both intracellular (RBC) and extracellular. As for the former, the concentration of RBC GSSG, the levels of Hb and membrane proteins S-glutathionylation, and the carbonylated protein levels were considered. Instead, at the plasma level, MDA levels, total thiols (LMW-SH + LMW-SS) released from cells and tissues were considered and, finally, PTI was calculated. From Figure 7 shows that, already within the first 30 min of oxidative stimulus, intracellular levels of GSSG increase abruptly. The parameter stabilizes over time (considering the constant stimulus provided by the settings of the experiment) but it is possible to note that two other parameters also go parallel with the increase of the GSSG. S-glutathionylation on erythrocyte proteins (Hb and membrane) gradually increases over time as a result of

those events aimed both at decreasing intracellular levels of GSSG (by thiol/disulfide exchange with PSH) and protecting the proteins cysteinyl residues from irreversible modifications. At the extracellular level, however, there is only a slight increase, over time, in the production of the by-product of lipid peroxidation MDA, the values of protein carbonylation and PTI, remain almost constant due to the buffering action of the high PSH concentrations at extracellular level. Overall, it is shown that the plasma values measured in the clinical study are not related to oxidative stress but are therefore an independent variable and can instead be evaluated for possible metabolic correlations.

3.2-Correlations

Possible correlations between the various redox forms of blood thiols and tHCys measured in the clinical study were then evaluated to analyze which parameters are most closely associated. Table 4 (Appendix) shows that in most cases the parameters are unrelated and that the association between them is present only in some cases (Figures 8, 9, and 10).

In Figure 8, the values of tHCys and PTI (RSSP/PSH ratio) were analyzed. Our results show that there is a direct correlation between these two parameters: PTI value is directly proportional to RSSP levels compared to PSH ones, tHCys is particularly influenced by the concentration of protein mixed disulfides (Figure 6) and, as a result, if RSSP increases tHCys has the same behavior.

The correlation between tHCys and LMW-SH/LMW-SS data was then evaluated (Figure 9). It can be shown that, when plasma thiol concentrations are higher than disulfides, tHCys has lower values. Overall, plasma thiols (-SH) appear to affect tHCys levels. Figure 10 analyzes the correlation between tHCys and various plasma thiols (Cys, CysGly, GSH, and GSH+CysGly) and, from the results of the analysis, tHCys decreases when various plasma thiols have high concentrations.

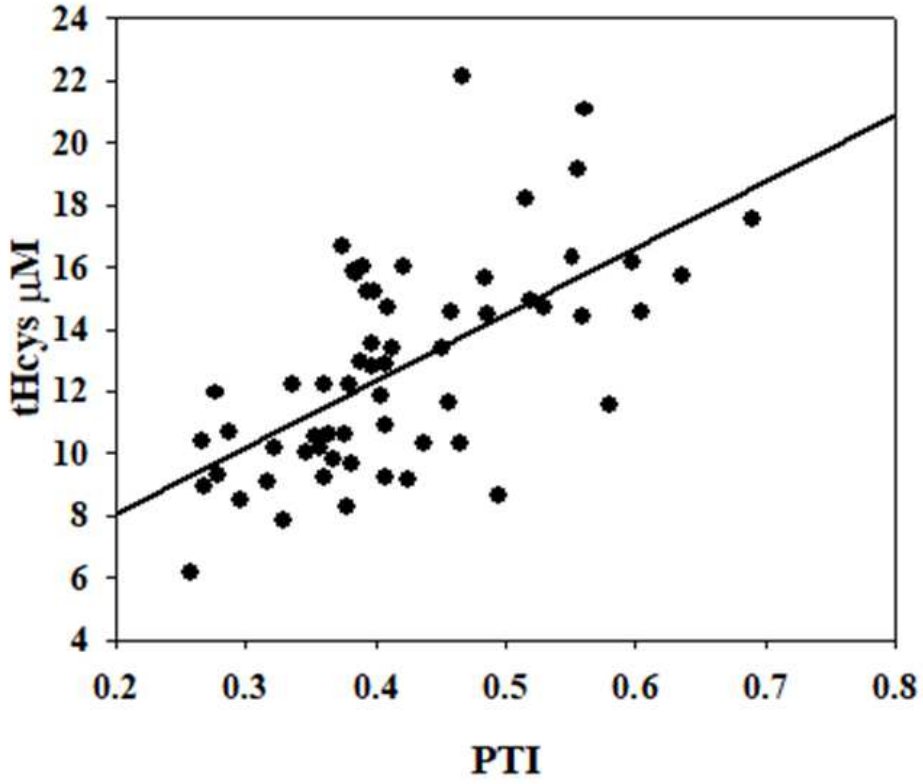


Figure 8. **Direct correlation between total homocysteine (tHCys) and protein thiolation index (PTI).** The parameters were measured in the plasma of healthy people enrolled in the clinical study.

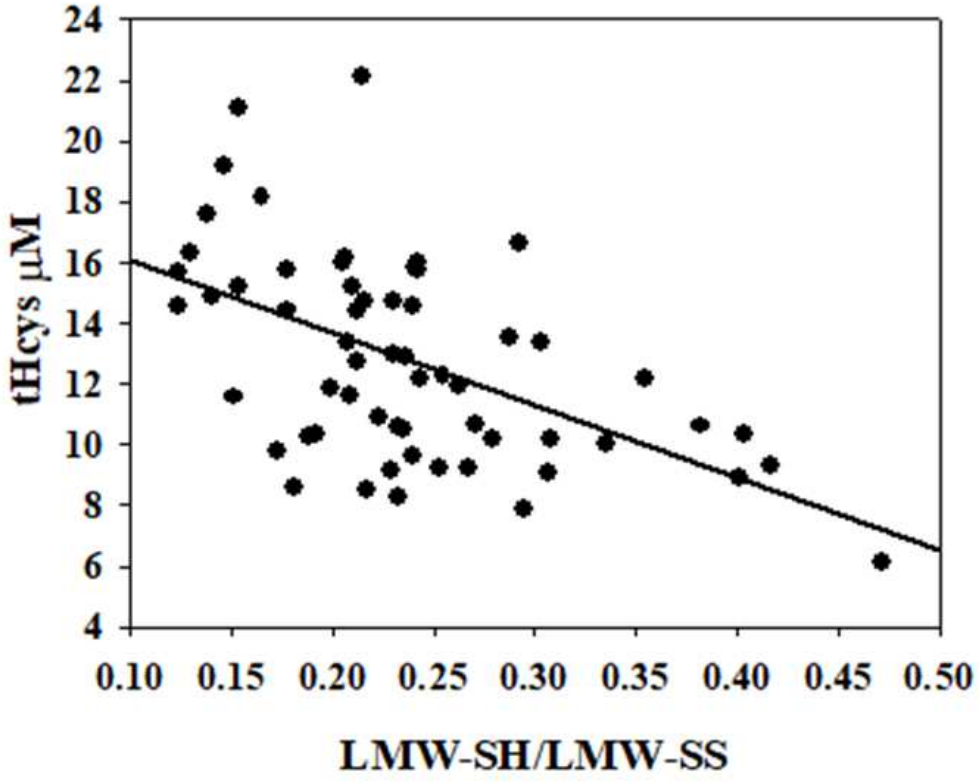


Figure 9. **Inverse correlation between total homocysteine (tHCys) and the ratio between low molecular weight thiols (LMW-SH) and low molecular weight disulfides (LMW-SS).** tHCys = HCys+ 2xCyss. The parameters were measured in the plasma of healthy people enrolled in the clinical study.

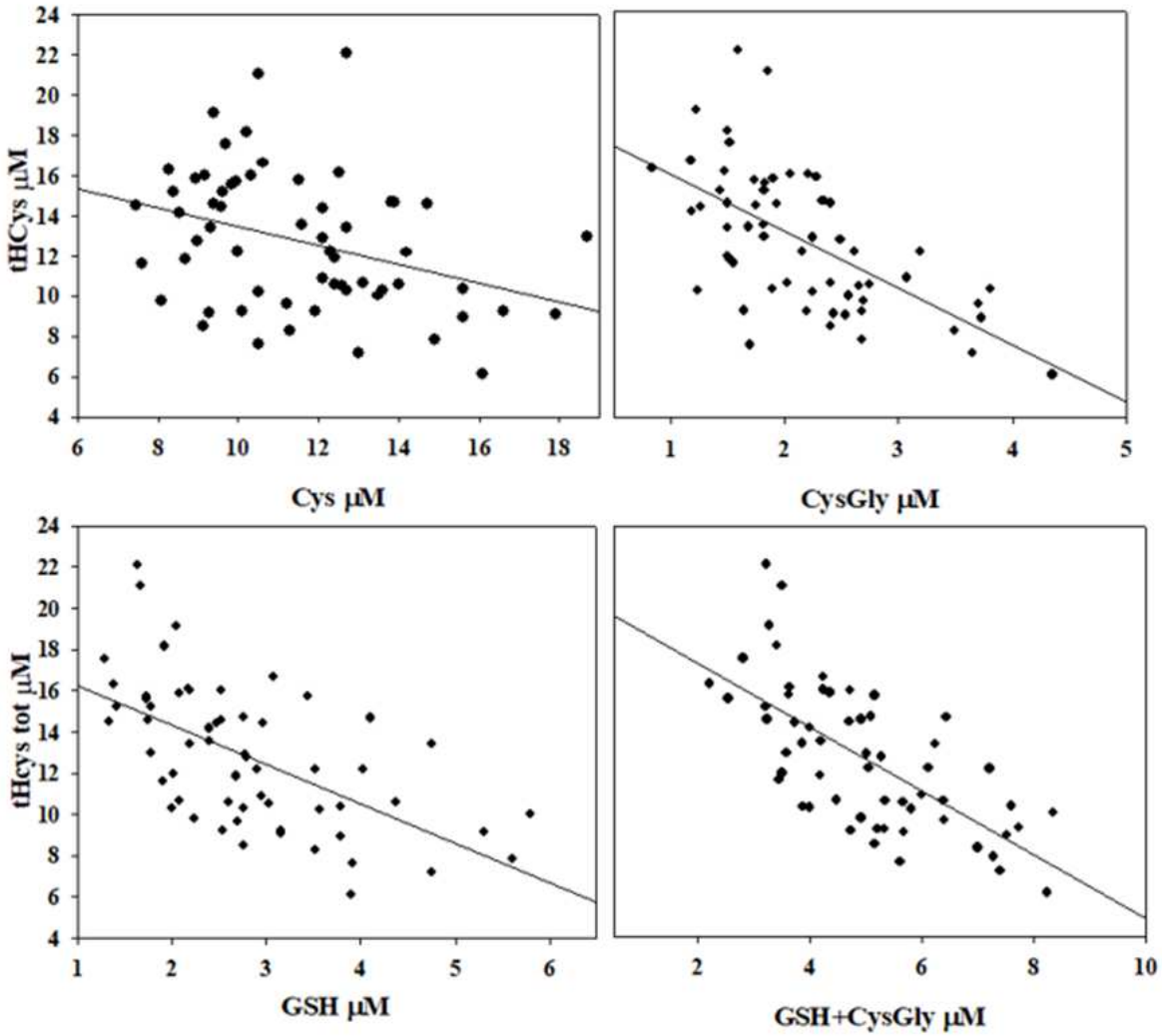


Figure 10. **Inverse correlation between total homocysteine (tHCys) and plasma low molecular mass thiols.** The parameters were measured in the plasma of healthy people enrolled in the clinical study.

Among plasma thiols, GSH has a pivotal role in influencing tHCys concentrations. From this data it can be seen that, as the levels of GSH increase in plasma, tHCys decrease and vice versa. These data support the hypothesis that by increasing GSH in plasma it is possible to decrease HCys levels favoring the release by plasma proteins and increasing the metabolism and/or excretion.

3.3-The contribution of cells/tissues to plasma GSH

We next evaluated if and how cells and tissues contribute to the extracellular content of GSH. GSH efflux outside the cell is mediated by carrier-dependent facilitated mechanisms. At the tissue level, GSH is then hydrolyzed by the combined action of membrane enzymes (γ-GT and dipeptidase) releasing Cys and other potential precursors that can be used to re-synthesize GSH and/or proteins at both proximal (intra-organ) and distal levels (inter-organ). GSH, Cys, and CySS plasma pools are highly dynamic: plasmatic GSH is rapidly degraded to Cys that can be taken locally or in more distant districts, Cys is also rapidly oxidized to CySS which is in turn internalized from different tissues, except for the liver. Plasma GSH, therefore, is highly necessary to keep Cys available for those tissues that cannot carry CySS internally (145, 146). We measured the release of GSH, if any, from both several lines of cultured cells and from the blood's main cellular components (Figure 11). The data are integrated with the values of the rate of GSH released from the kidney and liver, measured by models of isolated perfused rat organs. These analyses were carried out previously in our laboratory and agree with the data obtained by cell studies. We can observe that all the tested cells/organs release GSH with time. In these experiments, tGSH was measured because the free thiol undergoes auto-oxidation with time, therefore, if we measured only the LMW-SH, the amount of released GSH would be underestimated. Data obtained from RBCs confirm our previous results indicating that these cells are a physiological source of GSH for extracellular fluids (147). Erythrocytes were shown to be able to export mainly GSH and, in smaller quantities, HCys and Cys. The molecular mechanisms that generate this efflux of GSH by RBC are still unknown, but it is assumed that all those conditions both physiological and pathological that induce an increase/decrease in the levels of GSH within the erythrocytes or that modify the number are reflected in the plasma pool of GSH. As, similarly, it could influence the pool of plasma GSH also the malfunction of the membrane mechanisms aimed at transporting GSH outside the RBC as a result of pathophysiology and/or drug treatments. Plasma export by all these sources of LMW-SH and GSH is supposed to affect the plasma thiol/disulfide balance through thiol/disulfide exchange reactions. In particular, it may influence the levels of plasma tHCys.

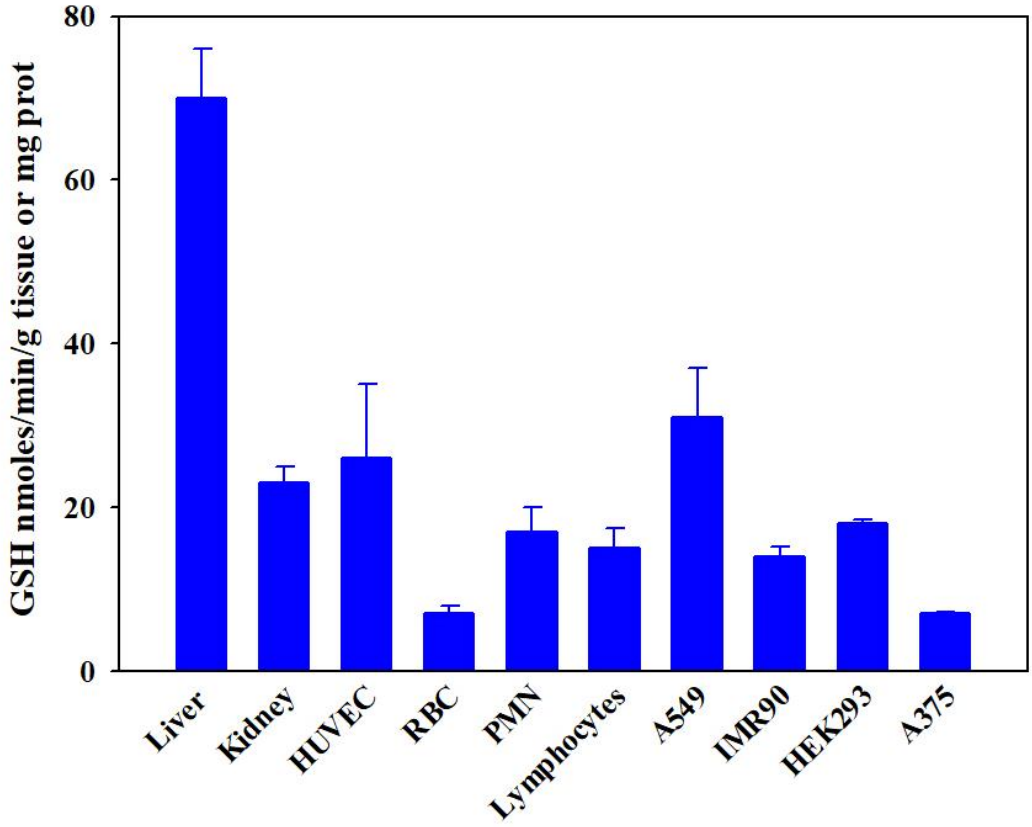


Figure 11. **Cells and tissues release of GSH.** Several line cells were cultured and, at confluence, tested for their ability to release tGSH to the extracellular milieu. RBCs, lymphocytes, and polymorphonuclear cells (PMN) were purified from human blood and freshly tested for their ability to release total GSH. Data about kidney and liver release were obtained by the isolated perfused organ model (95).

This hypothesis is straightened by our previous analysis carried out in the plasma of both several animal species and humans. These data are summarized in Figure 12 where the levels of tHCys are shown in relationship with the plasma levels of GSH + CysGly. Data from humans comprise also the values we obtained in the present study. As we can see the range of physiological levels of both GSH + CysGly and tHCys are different among the different animal species tested. For example, turkey and sheep have similar concentrations of plasma GSH+CysGly with humans between the ages of 60-80 (about 5 μM) but have higher levels of tHCys (between 25 and 30 μM). The calf, on the other hand, has very low plasma GSH+CysGly compared to the other species considered and has levels of tHCys much higher than the rest of the species analyzed. Additionally, there are also some differences among different strains of the same laboratory animal (see mouse ICRA vs ICRB and C, or Wistar and Sprague Dawley rats). It can also be

highlighted how the relationships between GSH+CysGly and tHCys can differ even within the same species. The different human age groups are a clear example to support this statement, younger individuals (20-40 years) have higher levels of GSH+CysGly and lower concentrations of tHCys than older subjects (60-80 years). In any case, higher are the levels of GSH + CysGly, lower are the levels of tHCys. Probably, this phenomenon is mainly influenced by the amount of GSH exported in each species. CysGly has been considered in our analysis because, while other thiols like Cys may derive from various sources including GSH decomposition, dietary intake, or intracellular reduction of circulating CySS, CysGly can only derive from GSH, through the hydrolysis processes to which the GSH is subjected on the external cell surface by γ-GT.

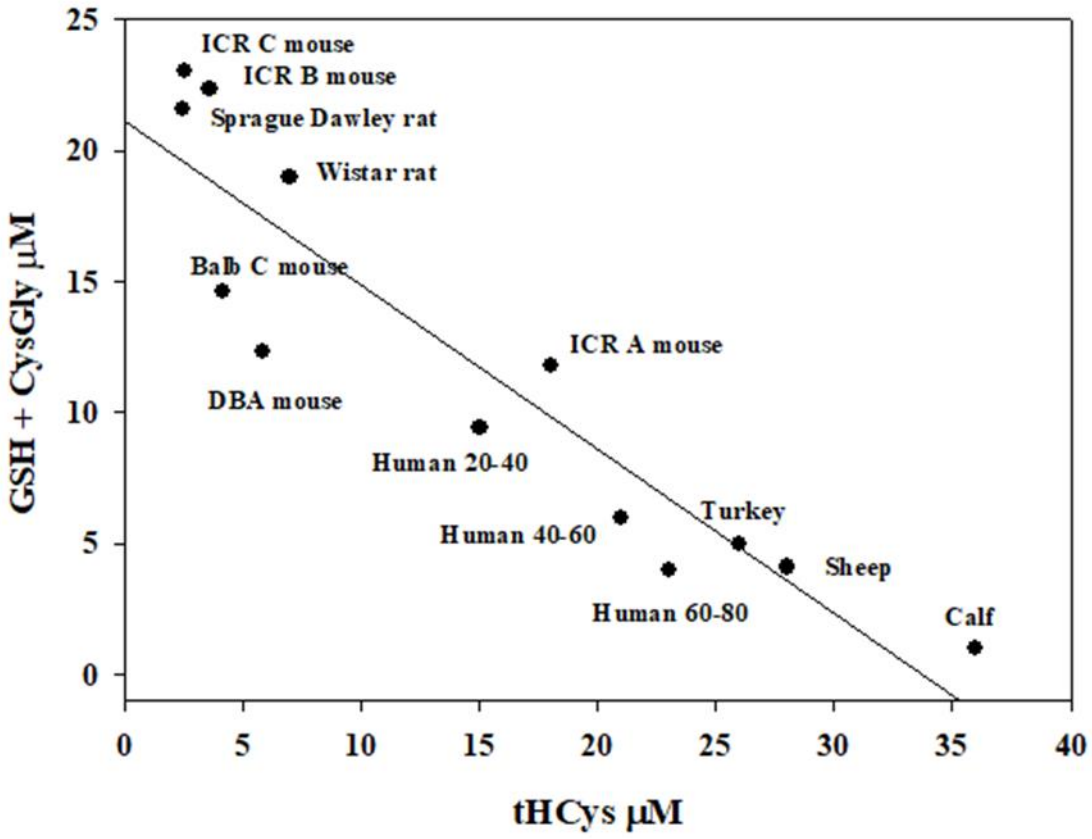


Figure 12. **Inverse correlation between GSH+CysGly content and tHCys in plasma of different species.** Data on laboratory animals were obtained by previous publications (33, 148, 149). The values of humans comprise original data from this work and previous data (45).

According to these observations, we hypothesize that if we can increase the release of GSH from cells/tissue this, may, favor the decrease of tHCys in plasma. We therefore evaluated if the treatment of cells with GSH enhancing molecules may increase this export. For these experiments, we selected two compounds: NAC and its ethyl ester NACET. NAC was selected since it is worldwide recognized as one of the most common ways to increase intracellular GSH (113, 108). NACET was selected since it is a new chemical entity that is supposed to be more efficient than NAC in increasing intracellular GSH given its better membrane permeating properties (101). In addition, since both NAC and NACET contain a free SH group they may also directly (by thiol-disulfide exchange reaction) decrease the tHCys in plasma (by reducing the disulphide bridges).

3.4-NACET reactivity compared with other thiols

The reactivity of the SH group of both NAC and NACET in comparison with that of other common LMW-SH (MESNA, Cys, and GSH) obtained using the Ellman assay (124) is presented in Figure 13.

In this comparative study we measured the reactivity of the -SH groups of some physiological LMW-SH (GSH and Cys) and of some currently used GSH donors (NAC and MESNA). MESNA is a thiol compound analog to taurine used to reduce the protein-bound HCys (150, 89).

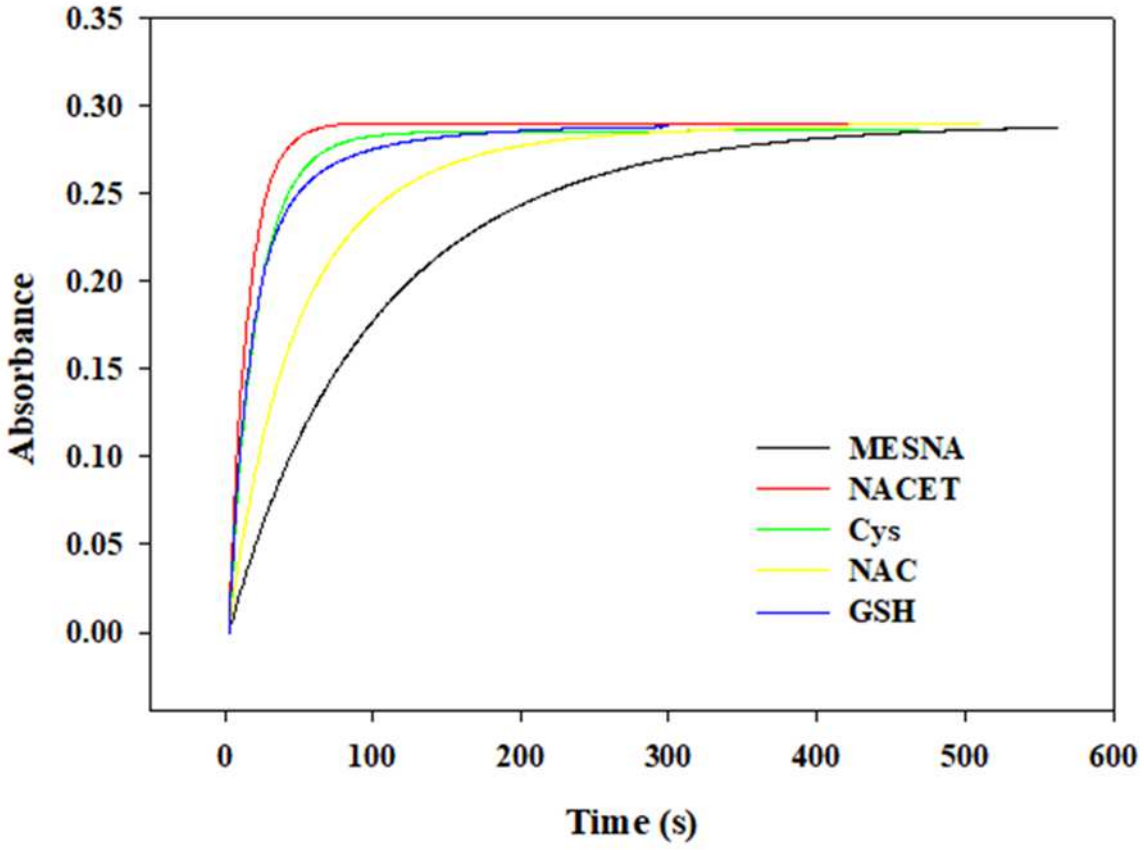


Figure 13. **Reactivity of the -SH group of sodium 2-mercaptoethanesulfonate (MESNA), N-acetylcysteine ethyl ester (NACET), cysteine (Cys), N-acetylcysteine (NAC) and glutathione (GSH)**. Equimolar solutions of the LMW-SH were reacted with Ellman's reagent and analysed at 410 nm wavelength by the spectrophotometer.

The shape of the titration curve indicates that the new chemical entity NACET is more reactive than the other LMW-SH. It is to underline that the -SH group of NAC has a quite lower reactivity ($7.41\pm0.15x10^5$) compared to NACET ($18.4\pm0.1x10^5$) and Cys ($13.6\pm0.2x10^5$). Therefore, NAC esterification appears to be associated with a considerable increase in -SH group reactivity. NAC and Cys, direct precursors of GSH, have a significant reactivity compared to, for example, MESNA ($2.18\pm0.11x10^5$). The constant rate of the velocity of the reactions reported in Table 5 gives a quantitative indication of this aspect. These preliminary data indicate NACET as a possible better agent to reduce plasma tHCys.

Thiol compound	K_2 (M^{-1} s^{-1})
MESNA	$2.18 \pm 0.11x10^5$
NACET	$18.4 \pm 0.1x10^5$
Cys	$13.6 \pm 0.2x10^5$
NAC	$7.41 \pm 0.15x10^5$
GSH	$6.25 \pm 0.16x10^5$

Table 5. Second order constant reaction rates between thiols and DTNB. Kinetic analyses were calculated by using the data obtained from the experiment reported in Figure 13.

3.5-Cells treated with NAC and NACET

To evaluate the ability of NACET to increase intracellular GSH concentration and the eventual effect on extracellular total HCys cultured cells (HeLa and HUVEC) were treated with this compound. Comparative experiments were performed by NAC and with vehicle-treated cells. Cellular lysates were used to assess intracellular levels of GSH and the culture medium for collected for the analysis of extracellular thiols. The collection of medium and cells was performed at 0, 4, 8, 12, and 18 hours. The analysis of thiols both within and outside cells was carried out by HPLC. A new method was developed able to measure by a single run all the LMW-SH of interest (Cys, GSH, NAC, and NACET, Figure 14). Both NAC and NACET are categorized as cysteine pro-drugs in that, once enter the cells they release Cys by enzymatic metabolism (de-esterase, de-acetylates) (101). Therefore, by the application of this separation method we could measure at each time the intracellular levels of both the parent compounds and levels of Cys (the rat limiting step precursor for GSH synthesis) and GSH. It is also evidence that NACET intracellular metabolism forms NAC first (by de-esterification) and then cysteine, by NAC de-acetylation.

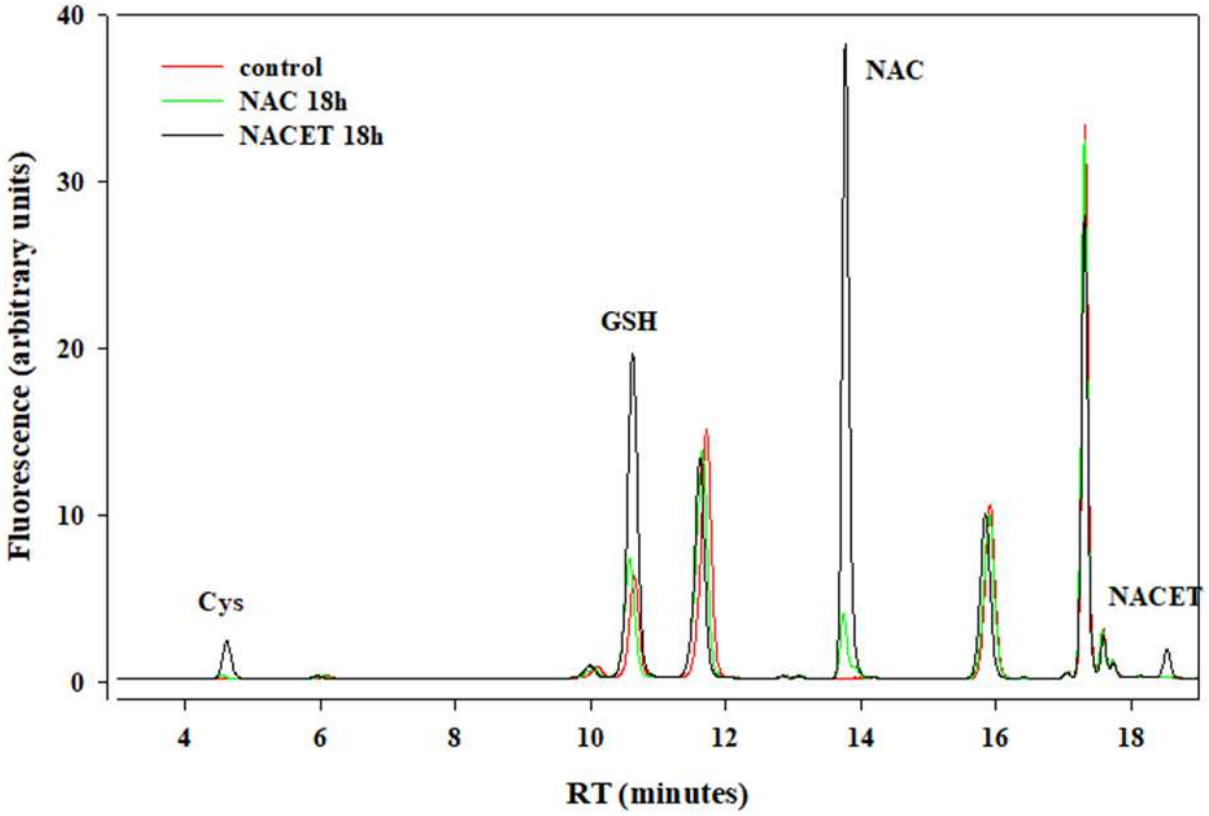


Figure 14. Chromatogram of cellular lysates obtained by HPLC separation. Lysates of HeLa cells treated with NAC or NACET were incubated with monobromobimane for fluorometric detection of thiols. An exemplificative tracing for each treatment after 18 hours is shown.

In Table 6, the intracellular values for each compound in the experiment with the HeLa cells are reported. The most interesting effect can be seen in cells treated with NACET regarding the levels of GSH which, compared to NAC, are much more pronounced. The increase already is evident after 4 hours of treatment and the GSH levels are maintained high all over the experiment. Instead, the treatment with an equimolar dose of NAC was not able to significantly increase the levels of GSH. There is also evidence that higher intracellular levels of NAC were obtained in samples treated with NACET compared to those treated with NAC. These data confirm that NACET is a better permeating agent than NAC.

Time points	Cys nmol/mg prot	GSH nmol/mg prot	NAC nmol/mg prot	NACET nmol/mg prot
NAC treatment				
0	0.125±0.03	91.9±4.9	-	-
4	0.096±0.02	88.9±5.4	0.0126±0.002	-
8	0.159±0.04	84.8±4.6	2.93±0.14	-
12	0.133±0.02	92.3±7.5	7.32±0.88	-
18	0.146±0.03	89.5±5.6	15.2±6.41	-
NACET treatment				
0	0.269±0.04	91.4±2.6	-	-
4	0.358±0.02	127±5	65.9±7.0	26.8±3.33
8	3.56±0.14	175±11	139±16	25.9±2.58
12	5.68±0.32	179±16	195±24	21.3±2.70
18	9.12±1.03	191±20	281±33	23.6±3.81

Table 6. Intracellular concentrations of the various compounds examined (Cys, GSH, NAC, and NACET) at different time points (0, 4, 8, 12, 18 hours) in HELA cells treated with 0.2 mM NAC or NACET. Data are the means±SD of three independent experiments.

Figures 15 and 16 show trends in intracellular GSH concentrations in response to treatments with 0.2 mM NACET or NAC. For both cell types, HeLa and HUVEC, it is possible to highlight how the best GSH enhancer effect is given by NACET already after 4 hours of administration.

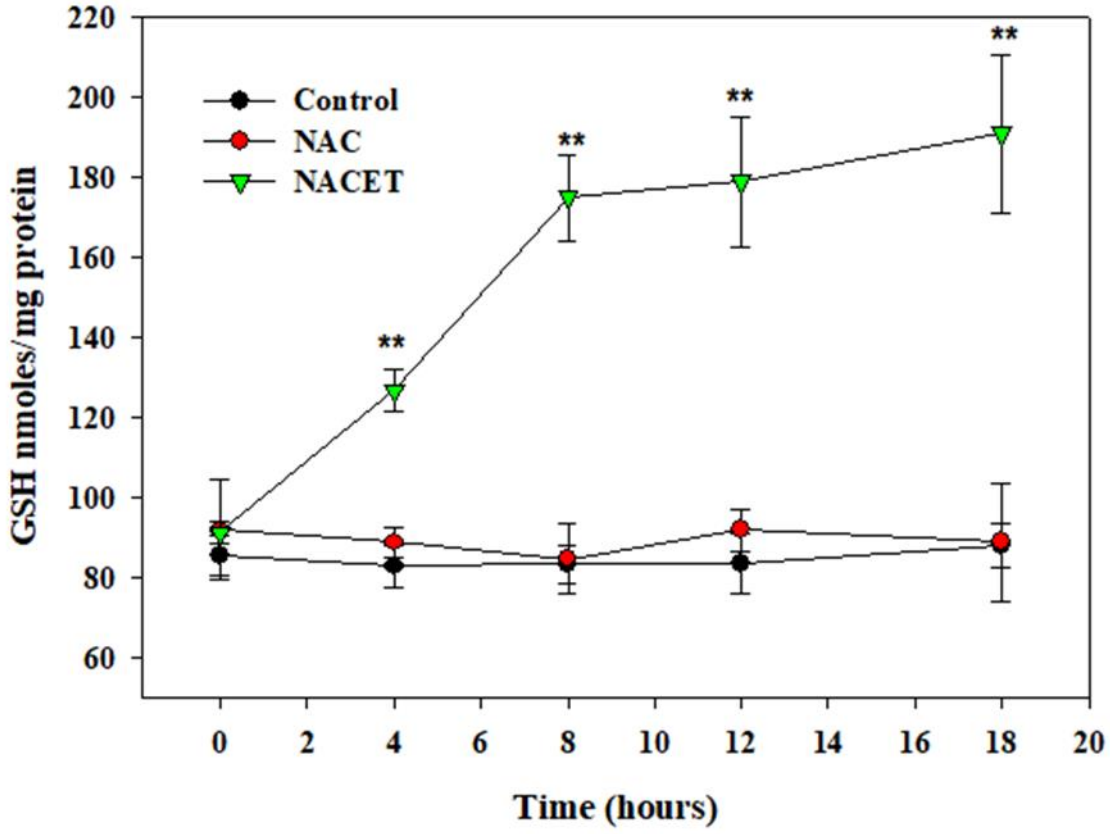


Figure 15. Intracellular GSH levels in HeLa cells treated with 0.2 mM NACET or NAC. Collecting time points at 0, 4, 8, 12 and 18 hours. Medium was used for control samples. Data are the means±SD. **p<0.01 vs 0 time.

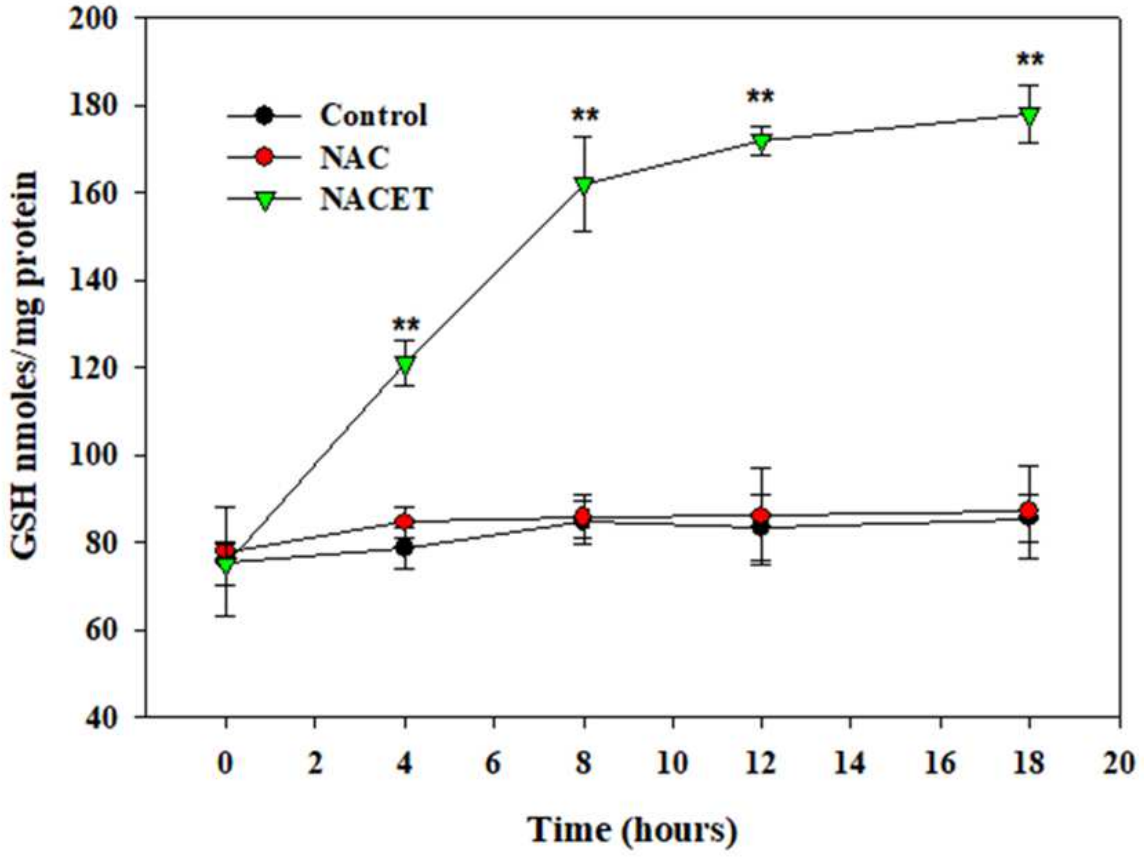


Figure 16. Intracellular GSH levels in HUVEC cells treated with 0.2 mM NACET and NAC. Collecting time points at 0, 4, 8, 12 and 18 hours. Medium was used for control samples. Data are the means±SD. **p<0.01 vs 0 time.

Figures 17 and 18 show trends in the GSH concentrations measured in the HeLa and HUVEC cell culture medium, respectively. It can be shown that in both cell models there is a significant increase in the release/efflux of GSH from the intracellular compartment to the extracellular one only in cells treated with NACET. Only a slight physiological release of GSH was observed for NAC treated cells, in analogy to control samples. These data indicate that cells release GSH in a more pronounced amount when are overloaded with a glutathione enhancer. In our experiments, only NACET can effectively contribute to the increase in intracellular GSH levels compared to equimolar NAC doses. In other studies (151) NAC can provide this effect which is probably due to the high concentrations of administration to which the cell cultures have been subjected. A similar result was highlighted in a work of our research group (101) in which high doses of NAC helped to increase the amounts of intracellular GSH. Finally, it should be noted that NAC concentrations administered in cell culture studies are at non-pharmacological doses and used to describe the antioxidant effect of the compound.

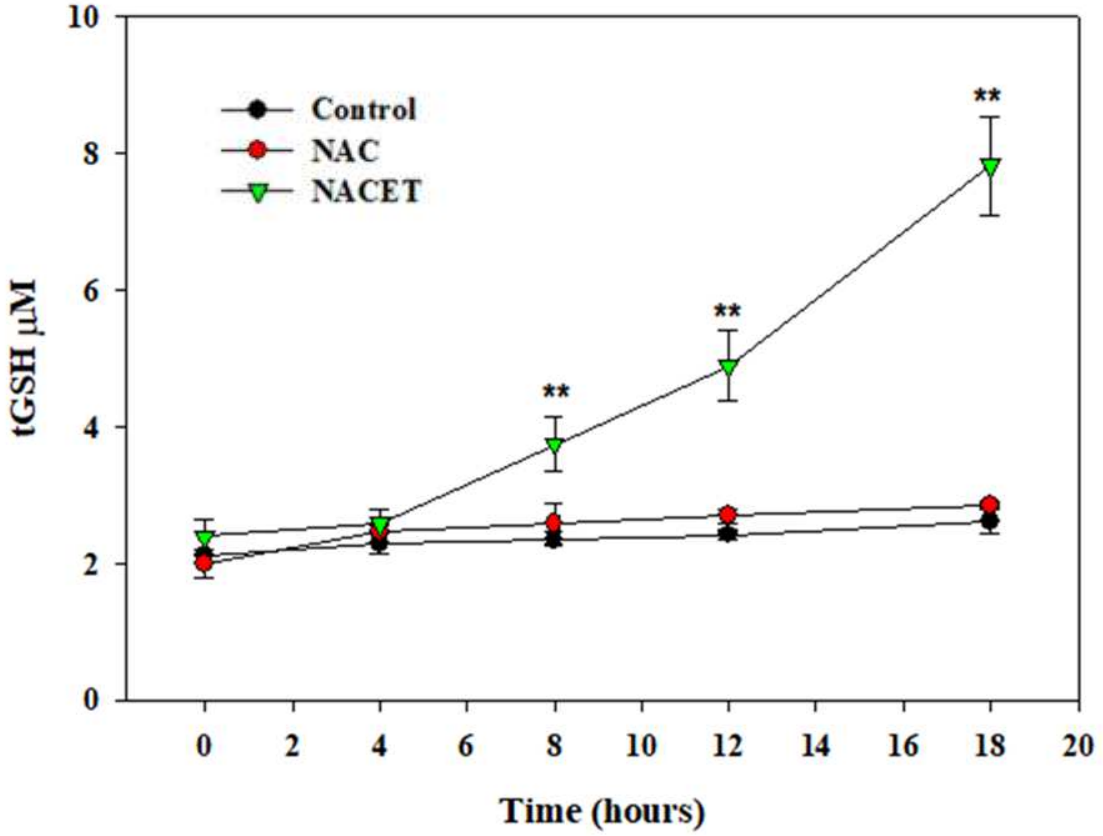


Figure 17. Extracellular concentrations of total glutathione (tGSH) in HeLa cells treated with 0.2 mM of NACET or NAC. Collecting time points at 0, 4, 8, 12 and 18 hours. Medium was used for control samples. Data are the means ± SD of three independent experiments. Data are the means±SD of three independent experiments. **p<0.01 vs 0 time.

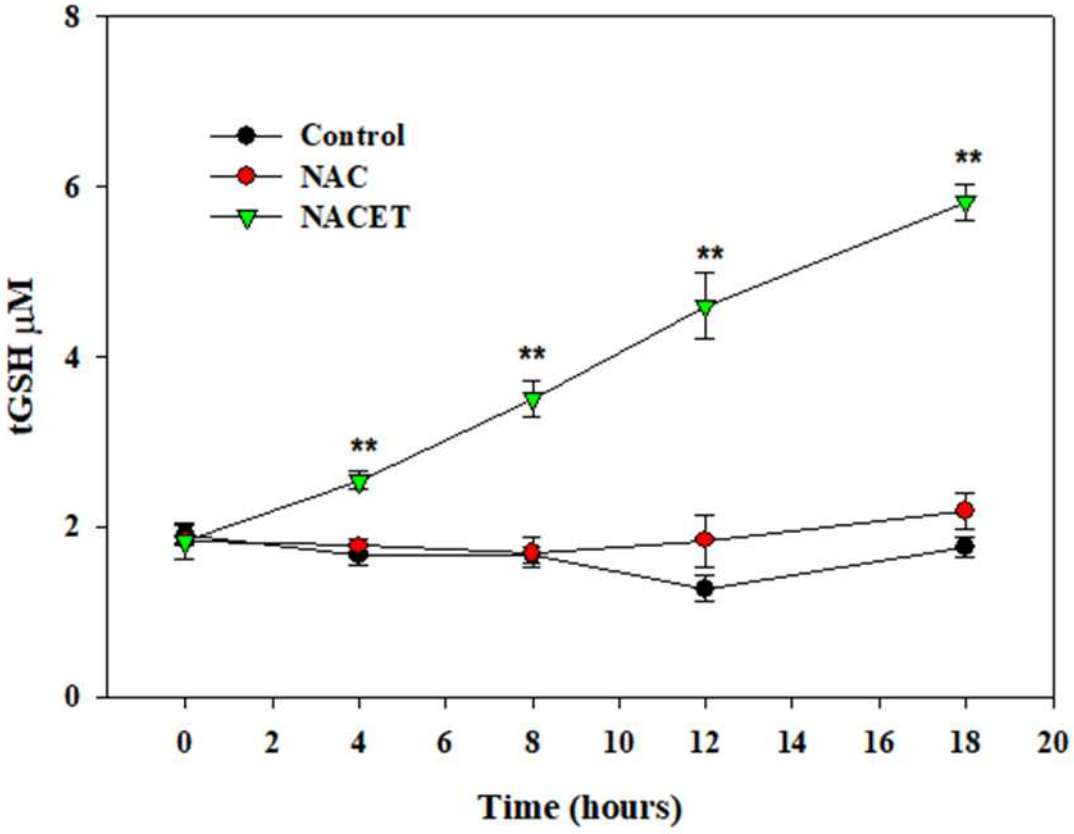


Figure 18. Extracellular concentrations of total glutathione (tGSH) in HUVEC cells treated with 0.2 mM of NACET or NAC. Collecting time points at 0, 4, 8, 12 and 18 hours. Medium was used for control samples. Data are the means±SD of three independent experiments. **p<0.01 vs 0 time.

3.6-Treatment with NACET and probenecid

It is of interest to evaluate which kind of membrane transporter is involved in GSH release from the cells. This aspect, to our knowledge, is not known. To this aim we tested the potential involvement of anionic transporters for this process by treatment with probenecid.

In some experiments, HeLa cell cultures were pre-treated with 200 mM probenecid (final concentration) for one hour before treatment with NACET. Probenecid is a drug with uricosuric activity it increases the urinary elimination of uric acid by lowering its blood concentration. But in most cases, its effect is to decrease the renal secretion of several drugs (such as penicillin) by increasing their half-life (152). We aimed to analyze the mechanisms by which GSH is released at the cellular level trying to block an anionic channel with the drug. Intracellular and extracellular GSH levels were measured by comparing treated and non-probenecid cell cultures. Results are shown in Figures 19 and 20.

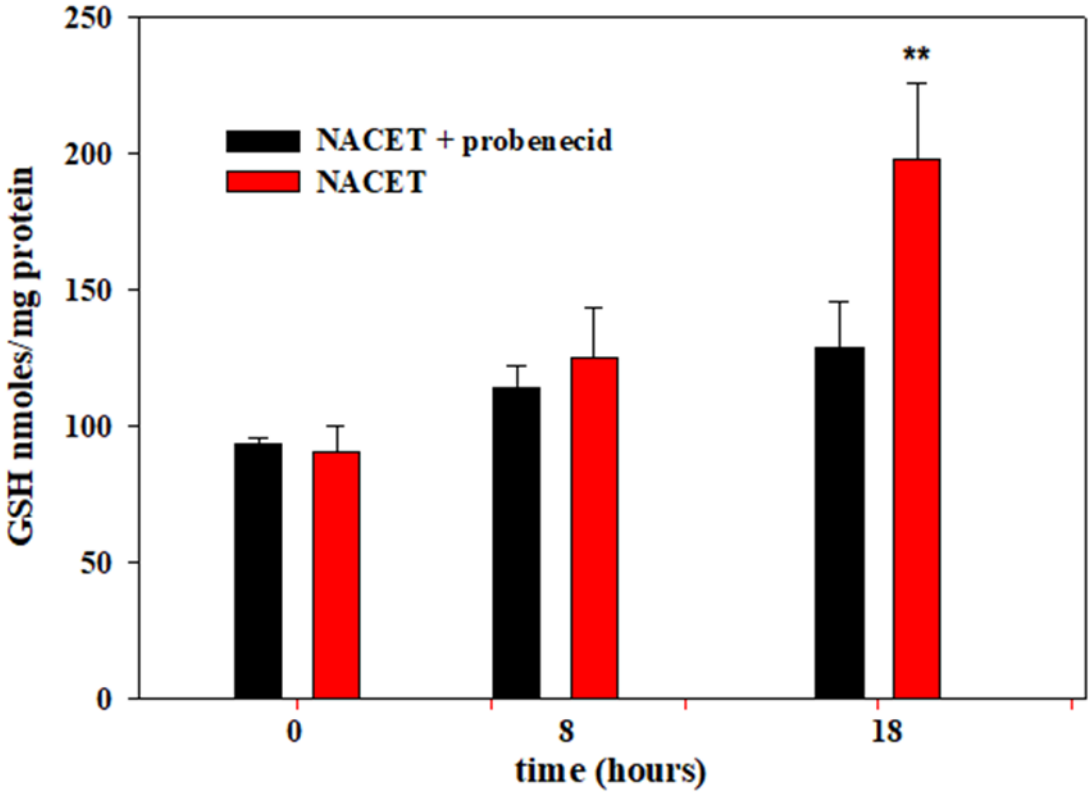


Figure 19. Intracellular glutathione (GSH) levels in HeLa cells treated with NACET or probenecid and NACET. Data are the means±SD of three independent experiments. **p<0.01 vs NACET.

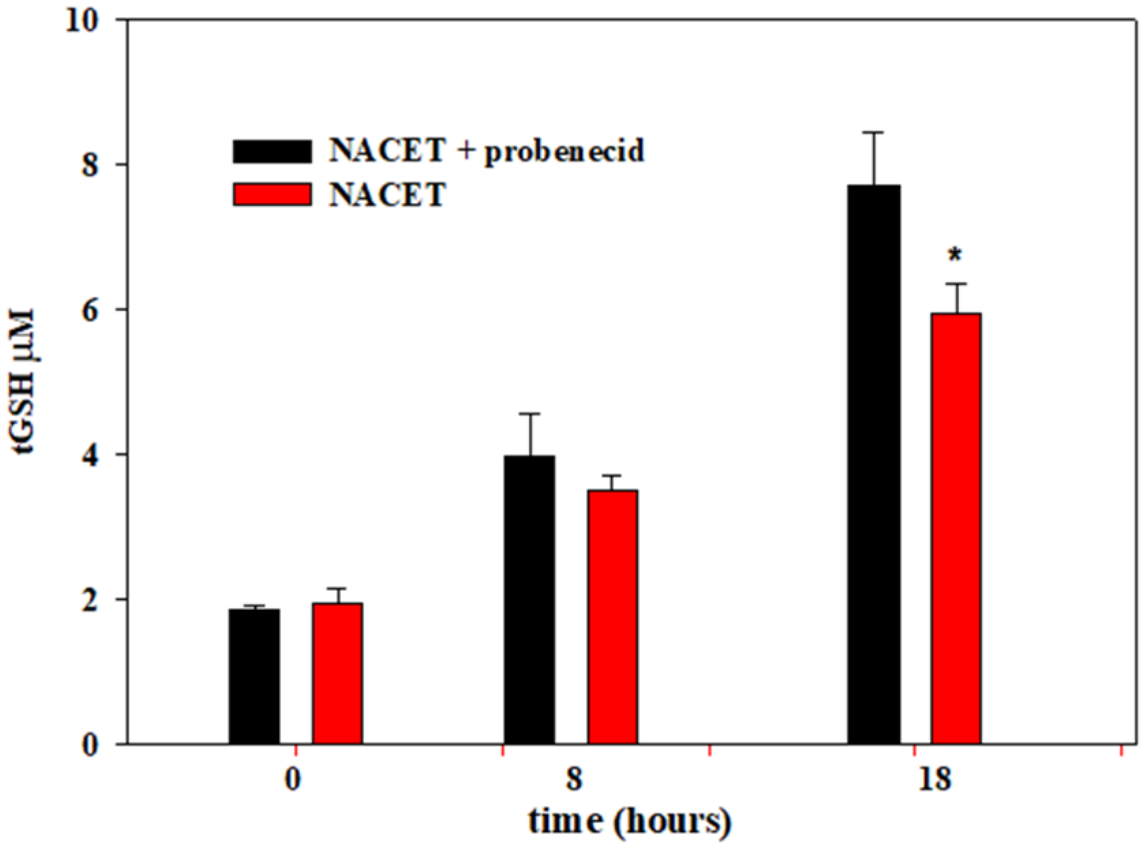


Figure 20. Extracellular total glutathione (tGSH) levels in HeLa cells treated with NACET or probenecid and NACET. Data are the means±SD of three independent experiments. *p<0.05 vs NACET.

Figure 19 shows that intracellular cells pre-treated with the drug show lower concentrations of GSH than untreated cells, which becomes more pronounced at 18 hours. At the extracellular level (Figure 20) the GSH concentrations measured in pre-treated probenecid cultures reflect, strangely, an increase in GSH efflux from the cell compartment. The data obtained therefore still leave doubts about the mechanism by which GSH is exported to the extracellular level and further studies are needed to describe the transport processes involving the tripeptide.

3.7-Human plasma treatment with LMW-SH

Finally, a series of experiments were performed to evaluate the reducing action that has various thiols on plasma HCySSP reduction. Plasma was treated for 8 hours (sample collection every hour) with equimolar doses (0.2 mM) of Cys, CysGly GSH, NAC, and NACET, results are shown in Figure 21.

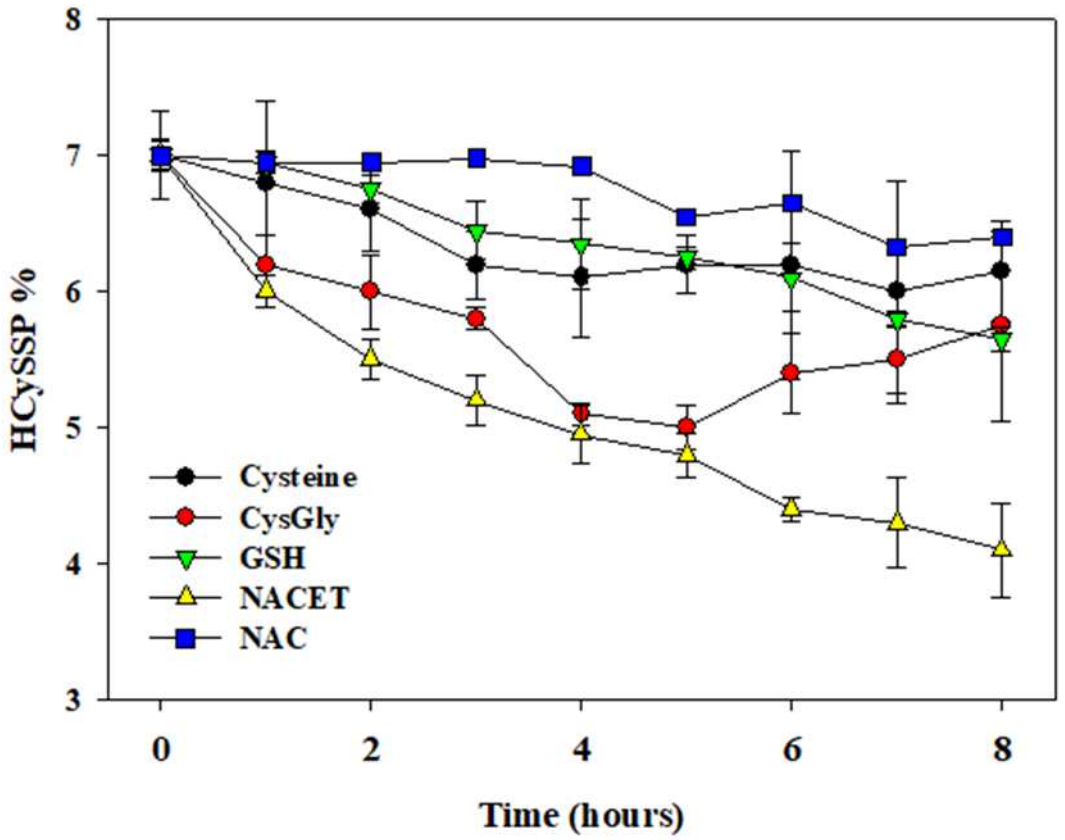


Figure 21. Reducing effect of several LMM-SH on the homocysteine bound as mixed disulphide to plasma proteins (HCySSP). Pooled human plasma was treated with various thiols (Cys, CysGly, GSH, NAC, and NACET) and at 1-hour intervals the levels of HCySSP were measured. Data are the means ± SD of three independent experiments.

Figure 21 shows that all the thiols examined are capable of releasing HCys from the thiol proteins present in the plasma. A drastic decrease in HCySSP can be seen in samples treated with NACET and hypotheses can be made. The reduction of the levels of modulated mixed disulphide from all the examined thiols can be given by their participation in thiol/disulfide exchanges with HCySSP, allowing the release of HCys. In addition, the assumption that NACET is able not only to act as a direct reducing agent (Figure 21) but to contribute to an effective increase in intracellular GSH levels

(Figures 15 and 16) should also be considered, this will allow greater efflux of GSH and, consequently, the reduction of other HCySSP molecules by GSH.

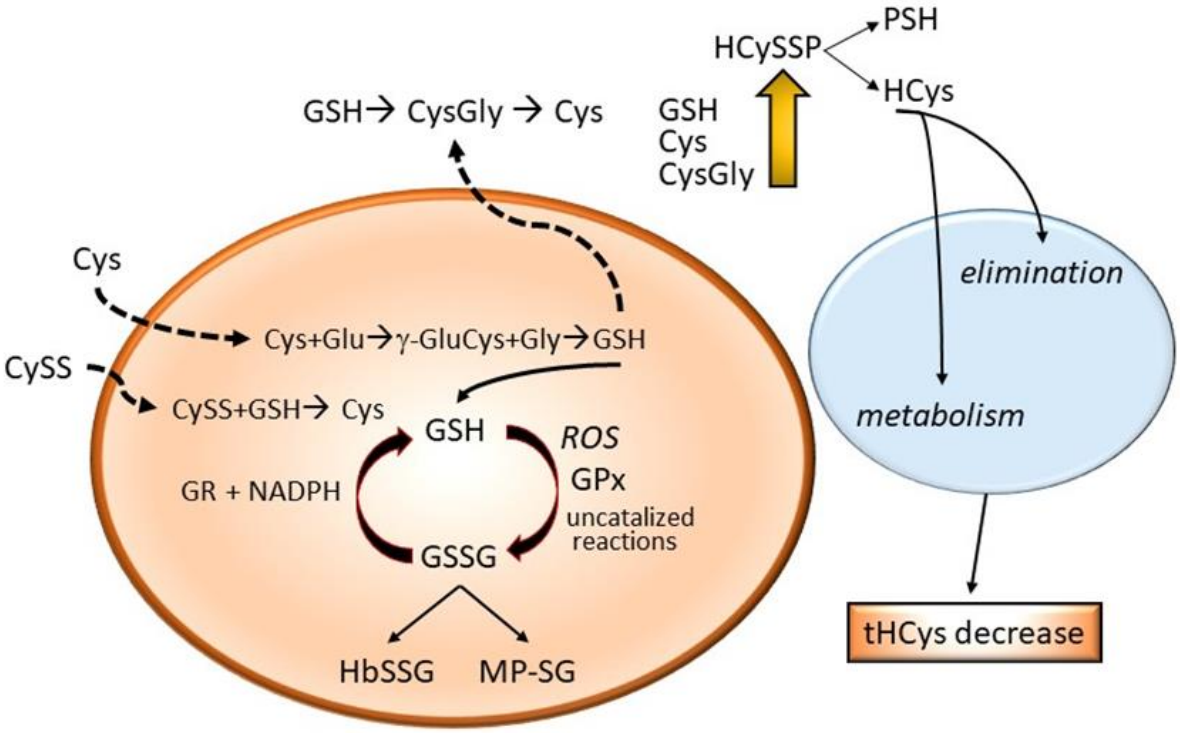


Figure 22. Proposed intracellular and extracellular pathways for GSH synthesis, release, and plasma HCys reduction. Cysteine (Cys), cystine (CySS), glutamate (Glu), γ-glutamyl cysteine (γ-GluCys), glycine (Gly), glutathione (GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione disulfide (GSSG), S-glutathionylated hemoglobin (HbSSG), S-glutathionylated membrane proteins (MP-SG), reactive oxygen species (ROS), glutathione peroxidase (GPx), cysteine glycine (CysGly), S-homocysteinylated proteins (HCySSP), protein thiols (PSH), homocysteine (HCys), total homocysteine (tHCys).

Conclusions

The most important observation made in this work is the identification of a new mechanism for lowering plasma homocysteine. Specifically, it was found that increasing GSH export can enhance the lowering of plasma homocysteine by decreasing the amount of homocysteine bound to proteins, thereby increasing the amount of eliminable homocysteine.

NACET, a new synthetic drug, proved to be the best agent able to induce this process both directly (by reducing thiol to disulfide) and indirectly (by increasing intracellular GSH synthesis and its export).

mHH is a condition in which plasma HCys levels are slightly higher than physiological concentrations (12-30 μmol/L range) (153) with subjects showing symptoms of vascular prevalence. The application of therapeutic approaches involving the supplementation of B vitamins to decrease HCys levels in circulation has yielded varying outcomes regarding their actual efficacy. Consequently, the scientific community has shifted its focus toward exploring alternative interventions that may offer more definitive results.

As analyzed by our research team in the study of the thiolome on healthy subjects, HCys is present in plasma mainly in three different forms: in reduced form as HCys, as HCySS, and, finally, as protein-mixed disulfide (HCySSP). The analysis we conducted showed that the most present species is HCySSP which represents the more stable fraction of this thiol, not being able to leave plasma. This redox form also prevails quantitatively in HH patients.

Furthermore, the correlations observed in our clinical study exclude an increase in mixed disulfides due to oxidative stress but show that increasing extracellular LMW-SH concentrations correspond to lower levels of HCySSP. We have also observed that many cells and tissues export GSH, and how an increase in GSH export, can significantly influence the redox balance of HCys.

Therefore, we looked for new pharmacological therapies that are effective in reducing HCys bounded to plasma proteins making it available again to be re-placed in the metabolic pathways that involve it (TSP and re-methylation cycle) or to be excreted at the renal level.

NAC, a mucolytic drug used as an emergency therapy for acetaminophen poisonings, has been studied as a pro-Cys drug to increase intracellular GSH levels (104) by the -SH group. In addition, NAC's ability to directly reduce HCySSP and contribute to lowering

HCys levels was also investigated (105). The action of NAC, however, is limited by some chemical characteristics of the molecule, the drug has both a low hydrophobicity and bioavailability, and, these factors, make it a difficult alternative to use therapeutically. Its pro-Cys action is determined by the Cys de-acetylation that takes place only once inside the cell and as described above, to allow a considerable increase in GSH levels at the intracellular level, high therapeutic doses that are difficult to implement are required. The object of this paper, NACET, has been evaluated as an alternative as well as, above all, its action in decreasing the plasma levels of HCys by both increasing the production of GSH and the direct reduction of HCySSP. Some characteristics of NACET were evaluated by comparing its action with that of other thiol compounds (including NAC). Our results showed that NACET has a higher reactivity than other compounds examined. To evaluate its enhancer action for GSH, *in vitro* experiments were carried out by treating cell cultures (HeLa and HUVEC) with NAC and NACET, aimed at measuring intracellular and extracellular levels of GSH and Cys, the end-product of both NACET and NAC metabolism. The results confirmed how NACET can penetrate better at the intracellular level; once inside the cell NACET is metabolized to NAC and, these concentrations, are higher than the concentrations of cell cultures treated directly with NAC. This results in a higher intracellular concentration of Cys in NACET treated cells and, as the concentration of Cys is a limiting factor for the synthesis of GSH, the higher the cysteine higher is GSH. Both in HeLa and in HUVEC cells, intracellular concentrations of GSH increased suddenly since the first hours after treatment with NACET confirming its high membrane permeability and its ability to act as Cys-pro drug. In comparative experiments NAC failed to give analogous results. The increase in intracellular GSH then caused a major efflux of GSH in NACET-treated cells. Some preliminary experiments were performed to identify the transmembrane carriers involved in GSH export. The results obtained by treating cells with the inhibitor of the organic anion transporter probenecid did not indicate the involvement of this transport mechanism. This aspect requires further investigation considering that plasma membranes contain a lot of different transporters.

In any case, the obtained data indicate that NACET can be a valuable drug for reducing HCys blood levels through the reduction of HCySSP mixed disulfide. This effect may result from the combined action of both the direct reducing activity of NACET against mixed disulfide and by enhancing the export of GSH.

In conclusion, NACET can be an optimal alternative to modulate plasma HCys levels but, more importantly, stimulate the synthesis and release of intracellular GSH helping to buffer the physio-pathological effects of oxidative stress both of physiological (aging) or pathological origin.

These data indicate that NACET is worth to be tested further in *in vivo* animal models of HH to evaluate if these promising *in vitro* results are confirmed.

Appendix

	GSH	GSSG	GSH/GSSG	Cys	CySS	Free Cys	Free HCys/HCySSP	tHCys	GSH+CG	PTI	HbSSG	RBC GSH	RBC GSSG	RBC GSH/GSSG	MP-SG	PCO	MDA
GSH	X	<0.01	<0.001	<0.01	<0.05	<0.05	<0.001	<0.001	<0.001	<0.01	Ns	Ns	Ns	Ns	Ns	Ns	Ns
GSSG	<0.05	X	<0.001	Ns	Ns	Ns	<0.05	<0.01	<0.05	<0.01	Ns	Ns	Ns	Ns	Ns	Ns	Ns
GSH/GSSG	<0.01	<0.001	X	<0.05	<0.05	Ns	<0.001	<0.01	<0.001	<0.01	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Cys	<0.05	<0.05	<0.05	X	<0.05	Ns	<0.05	<0.05	<0.01	<0.05	Ns	Ns	Ns	Ns	Ns	Ns	Ns
CySS	Ns	Ns	<0.05	<0.05	X	<0.05	<0.05	<0.05	<0.05	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Free Cys	Ns	Ns	<0.05	<0.01	<0.001	X	<0.05	<0.05	<0.05	<0.05	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Free HCys/HCySSP	<0.01	Ns	<0.01	<0.05	<0.05	Ns	X	<0.01	<0.001	<0.01	Ns	Ns	Ns	Ns	Ns	Ns	Ns
tHCys	<0.001	Ns	<0.01	<0.05	<0.05	Ns	<0.01	X	<0.01	<0.001	Ns	Ns	Ns	Ns	Ns	Ns	Ns
GSH+CG	<0.001	Ns	<0.001	<0.05	<0.05	Ns	<0.001	<0.001	X	<0.001	Ns	Ns	Ns	Ns	Ns	Ns	Ns
PTI	<0.01	Ns	<0.001	<0.01	<0.01	Ns	<0.01	<0.001	<0.001	X	Ns	Ns	Ns	Ns	Ns	Ns	Ns
HbSSG	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	X	Ns	Ns	<0.01	Ns	Ns	Ns
RBC GSH	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	X	<0.01	<0.001	Ns	Ns	Ns
RBC GSSG	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	<0.001	<0.05	X	<0.001	<0.001	Ns	Ns
RBC GSH/GSSG	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	<0.001	Ns	<0.001	X	<0.001	Ns	Ns
MP-SG	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	<0.001	Ns	<0.001	<0.001	X	Ns	Ns
PCO	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	<0.05	<0.05	<0.05	X	Ns
MDA	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns	<0.01	Ns	Ns	<0.01	<0.01	<0.05	X

Table 4. Statistically significant correlations between the various redox forms of blood thiols measured in the clinical study and biomarkers of oxidative stress. Ns: not-statistically significant.

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Comparison of Pollutant Effects on Cutaneous Inflammasomes Activation

John Ivarsson [1,†], Francesca Ferrara [2,†], Andrea Vallese [3,4], Anna Guiotto [3,5], Sante Colella [6], Alessandra Pecorelli [3,4] and Giuseppe Valacchi [3,4,7,*]

[1] Department of Food, Bioprocessing and Nutrition Sciences, Plants for Human Health Institute, NC Research Campus, NC State University, Kannapolis, NC 28081, USA; jcivarss@ncsu.edu
[2] Department of Chemical, Pharmaceuticals and Agricultural Sciences, University of Ferrara, 44121 Ferrara, Italy; frrfnc3@unife.it
[3] Department of Animal Sciences, Plants for Human Health Institute, NC Research Campus, NC State University, Kannapolis, NC 28081, USA; andrea.vallese@unife.it (A.V.); anna.guiotto@edu.unife.it (A.G.); apecore@ncsu.edu (A.P.)
[4] Department of Environmental Sciences and Prevention, University of Ferrara, 44121 Ferrara, Italy
[5] Department of Neuroscience and Rehabilitation, University of Ferrara, 44121 Ferrara, Italy
[6] Department of Biotechnology, Chemistry and Pharmaceutical Sciences, University of Siena, 53100 Siena, Italy; sante.colella@unisi.it
[7] Department of Food and Nutrition, Kyung Hee University, Seoul 26723, Republic of Korea
* Correspondence: gvalacc@ncsu.edu
† These authors contributed equally to this work.

Abstract: The skin is the outermost layer of the body and, therefore, is exposed to a variety of stressors, such as environmental pollutants, known to cause oxinflammatory reactions involved in the exacerbation of several skin conditions. Today, inflammasomes are recognized as important modulators of the cutaneous inflammatory status in response to air pollutants and ultraviolet (UV) light exposure. In this study, human skin explants were exposed to the best-recognized air pollutants, such as microplastics (MP), cigarette smoke (CS), diesel engine exhaust (DEE), ozone (O_3), and UV, for 1 or 4 days, to explore how each pollutant can differently modulate markers of cutaneous oxinflammation. Exposure to environmental pollutants caused an altered oxidative stress response, accompanied by increased DNA damage and signs of premature skin aging. The effect of specific pollutants being able to exert different inflammasomes pathways (NLRP1, NLRP3, NLRP6, and NLRC4) was also investigated in terms of scaffold formation and cell pyroptosis. Among all environmental pollutants, O_3, MP, and UV represented the main pollutants affecting cutaneous redox homeostasis; of note, the NLRP1 and NLRP6 inflammasomes were the main ones modulated by these outdoor stressors, suggesting their role as possible molecular targets in preventing skin disorders and the inflammaging events associated with environmental pollutant exposure.

Keywords: oxinflammation; inflammasome; pollutants; skin

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1. Introduction

Exposure to air pollutants is considered one of the predominant risk factors that can negatively impact human health. One of the primary organs affected by environmental stressors is the skin, which represents the main barrier against the external environment. Due to the skin's ability to induce inflammatory and oxidative stress reactions throughout the cutaneous tissue, exposure to environmental pollutants has been demonstrated to affect the skin barrier function, altering epidermal proteins and skin lipids and damaging DNA [1,2]. The interplay between oxidative and inflammatory mediators induced by pollutant exposure (oxinflammation) [3] may culminate in the alteration of cutaneous homeostasis, leading to the exacerbation of cutaneous inflammatory disorders (i.e., psoriasis, atopic dermatitis, etc.), as well as skin cancer and premature skin aging [4,5]. One of the

main environmental sources of exogenous cutaneous reactive oxygen species (ROS) is represented by UV, which, when absorbed by cutaneous chromophores (e.g., bilirubin, melanin, B6 vitamins, etc.), can promote the production of singlet oxygen ($^1O_2$) and superoxide radical anions ($O_2^{\bullet}$), which can indirectly damage DNA [6]. Regarding air pollutants, diesel engine exhaust (DEE), one of the principal sources of particulate matter (PM), and cigarette smoke (CS) are recognized to be among the most dangerous to human skin. However, in recent years, other recognized pollutants, such as tropospheric ozone (O_3) and microplastics (MP), have garnered attention as possible modulators of the inflammatory response in skin disorders [7,8]. The increasing industrialization and use of vehicles has played a major role in increasing the tropospheric O_3 concentrations in urban areas. Moreover, due to the growing consumption of plastics, MP are now considered a new class of atmospheric contaminants that are dangerous to human health [8]. Although air pollutants can exert similar effects on human skin, their different chemical natures confer upon them different mechanisms of action. For instance, both DEE and CS are composed of a gas phase and a solid phase, but O_3 or trioxygen can be mainly found in the gas state, and MP are represented by synthetic solid particles. The solid fractions of DEE are mainly composed of PM, a mixture of solid and liquid particles including poly-aromatic hydrocarbons, metals, and inorganic and organic toxins that, when deposited on human skin, are extremely reactive with the cutaneous components of the stratum corneum (cholesterol, fatty acids, and ceramides), triggering oxidative and inflammatory reactions [9]. Moreover, evidence has demonstrated that PM can even penetrate the skin, reaching the deep layers [10], especially when the skin is damaged [11]. CS is a complex aerosol composed of a mixture of more than 4700 chemical substances, among which 1014 are low-molecular-weight carbon- and oxygen-centered radicals [12,13] that can participate in oxidative events [14]. In particular, the main cause of oxidative stress reactions linked to CS is represented by the production of reactive aldehydes during lipid peroxidation events (4-hydroxy-2,3-nonenal and malondialdehyde) [15], or due to the presence of α,β-unsaturated aldehyde species (acrolein and crotonaldehyde) in the CS composition [16]. A similar mechanism of action is ascribed to O_3, which, although too reactive to penetrate the skin, can directly interact with skin biomolecules, such as lipids, proteins, or DNA, leading to the production of ROS and other nonradical species such as aldehydes (4-hydroxy-nonenal, 4HNE), which can promote the alteration of its structure and function [17,18]. MP, by contrast, originating from the degradation of plastic products, can reach a sub-millimetric size (5 µm or less) and thus be inhaled or ingested [19]. Once introduced into the organism, MP can trigger inflammatory and oxidative stress reactions, along with cytotoxicity and cell death (i.e., apoptosis), due to the presence in their composition of reactive chemicals (i.e., additives, phthalates, heavy metals, etc.) and their ability to carry pathogens [8,20,21]. Recently, it has been found that MP below 100 nm can penetrate human skin. Although the possible mechanism of action of MP within the cutaneous tissue is unclear, it might resemble the behavior displayed by PM [22]. In recent decades, exposure to air pollutants has been shown to modulate cutaneous inflammatory responses via the activation of a new class of inflammatory pathways, namely inflammasomes [23]. Of note, the activation of these multiprotein pathways has been correlated with the exacerbation of different skin conditions, including skin inflammaging [24,25]. The nucleotide-binding and oligomerization domain (Nod)-like receptors (NLRs) represent the most studied inflammasome families involved in the inflammatory response of the innate immune system in many pathological conditions. Upon sensing different stimuli, such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), these multiprotein complexes form a platform scaffold due to the assembly of the sensing NLR receptor and the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) [26]. The scaffold formation leads to the activation of the caspase-1 enzyme, which can proteolytically cleave pro-inflammatory cytokines IL-1β and IL-18, leading to their maturation and release, therefore promoting an inflammatory status and even a type of cell death called pyroptosis [27]. Among all NLR members (22 up to now), NLRP3, NLRP1, NLRC4, and NLRP6 represent the most studied inflammasomes,

involved in many inflammatory tissues pathologies, where NLRP3, NLRP1, and NLRC4 are mainly related to autoinflammatory skin disorders [4]. However, some studies are also exploring the possible involvement of NLRP6 in human skin homeostasis [28]. Although inflammasomes can be activated by a variety of stimuli, ROS have been addressed as one the main insults responsible for inflammasome activation [23].

The aberrant activation of inflammasomes has been correlated to the development of skin conditions such as psoriasis, atopic dermatitis, vitiligo, and many other cutaneous autoinflammatory syndromes [4,29–32]. Although air pollutants have been shown to differently modulate cutaneous inflammasomes, the role that outdoor stressors can have in the activation of different inflammasomes has never been investigated. Here, we used human skin biopsies to understand how prolonged exposure to air pollutants such as UV radiation, DEE, CS, O_3, and MP can differently affect specific markers related to oxidative stress, DNA damage, and skin aging. In addition, we explored how long-term exposure to a single environmental pollutant can modulate gene expression, scaffold formation within ASC, the release of inflammatory cytokines, and cell death due to pyroptosis, related to the major NLR inflammasomes (NLRP1, NLRP3, NLR6, and NLRC4) in human skin.

2. Results

2.1. Effect of Environmental Pollutants on Cutaneous Oxidative Stress and DNA Damage

To evaluate how skin explants' homeostasis could be affected by different air pollutants, we first exposed the human cutaneous biopsies to MP, CS, DEE, O_3, and UV for 1 or 4 days and the levels of oxidative stress markers (4-hydroxynonenal (4HNE), Sestrin 2 (SESN2)) and DNA damage (8-oxo-2′-deoxyguanosine (8-OHdG)) were determined. The list of the air pollutants exerting an effect on the abovementioned markers is reported in Supplementary Figure S1. Human skin biopsies exposed to the different pollutants displayed increased levels of 4HNE at both timepoints, after 1 day (DAY 1) and 4 days (DAY 4) of exposure (Figure 1a). Among all air pollutants, MP and UV seemed to particularly affect the production of 4HNE.

In addition, the levels of Sestrin 2 (SESN2), a highly evolutionarily conserved protein that, in response to various stimuli, controls many cellular responses related to oxidative stress, autophagy, DNA damage, metabolism and inflammation, were also assessed [33]. As depicted in Figure 1b, air pollutants, in particular MP, CS and DEE promoted the decrease of SESN2 levels after the first exposure (DAY 1), and this effect was even more evident at the later timepoint, (DAY 4), where all pollutants promoted a downregulation of SESN2. Since oxidative stress events can lead to DNA damage, the levels of 8-OHdG, one of the major products of DNA oxidation, were also measured. As shown in Figure 1c exposure to air pollutants promoted an increase in 8-OHdG levels, at both timepoints, 1DAY and 4DAY after the exposure. Along with UV radiations, that are known to be one of the main causes of DNA damage within the skin, also MP, CS, DEE and O_3 significantly promoted oxidation of DNA, especially at DAY 1, suggesting their involvement in a more acute response of the skin against the environmental insults. UV instead, exerted the greatest effect after a prolonged exposure time (DAY 4).

2.2. Exposure to Air Pollutants Promotes Extrinsic Aging Markers

The pollutants-induced extrinsic skin aging process [5], was evaluated by measuring the levels of the proteolytic enzyme metalloproteinase-2 (MMP2) and the possible degradation of Type I Collagen. The zymography assay revealed that exposure to the different air pollutants could promote the activation of MMP2 (Figure 2a), one of the main MMPs involved in extracellular matrix (ECM) degradation. Indeed, this technique can identify proteins with gelatinolytic activity based on their capacity to digest the substrate incorporated into the gel. Of note, all air pollutants promoted an increase in active-MMP2 expression levels at the end of the 4 days exposure, with O_3 and MP displaying the major effect. However, any significant change in MMP2 activation was detected at DAY 1 timepoint. As a result of MMP2 activation, the cutaneous levels of Type I Collagen in response

to the different pollutants was also measured (Figure 2b). As depicted in Figure 2b, MP and DEE significantly decreased Type I Collagen levels compared to unexposed tissues already after 1 day of exposure, whereas skin biopsies exposed to the other pollutants did not display any relevant changes although O_3 and CS showed a trend in the decrease of collagen protein levels. After 4-days exposure, DEE displayed the major effect on Type I Collagen loss, followed by UV and MP.

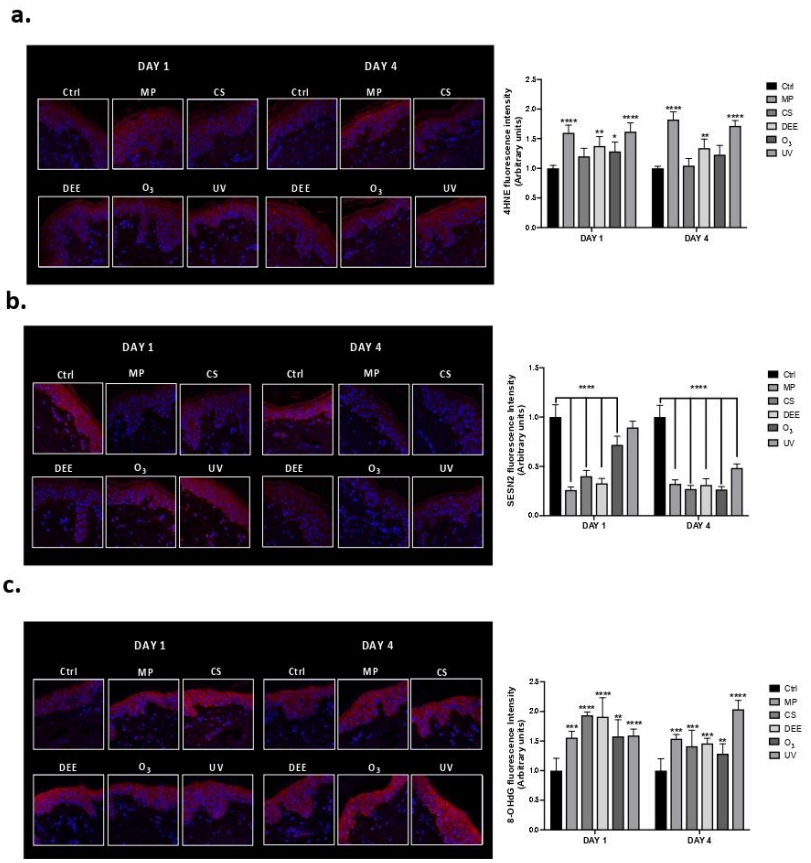

Figure 1. Immunofluorescence staining for 4HNE (**a**), SESN2 (**b**), and 8-OHdG (**c**) in human skin biopsies exposed to different air pollutants and collected at the indicated timepoints (DAY 1 and DAY 4). Red represents 4HNE (**a**), SESN2 (**b**), 8-OHdG (**c**) and the blue staining (DAPI) represents nuclei. Images were taken at 40× magnification and the fluorescent signal was quantified using ImageJ software 1.53a (Java 1.8.0_172). Data are the results of the averages of at least three different experiments, * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$ pollutants vs. Ctrl at the respective timepoints (DAY 1 and DAY 4) by 2-way ANOVA followed by Tukey's post-hoc comparison test.

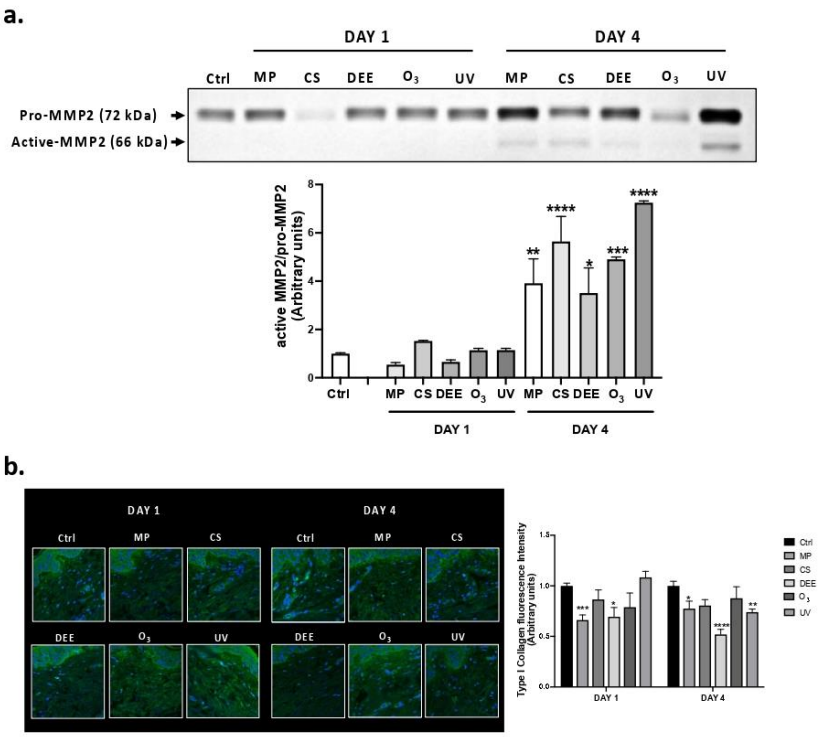

Figure 2. (**a**) Gelatin zymography for MMP-2 in human skin biopsies. The assay was performed on whole skin homogenates (10 µg) collected 1 day and 4 days after air pollutants exposure. The upper panel is representative of 3 independent experiments whereas the underneath graph represents the quantification of active-MMP2 over total pro-MMP-2 assessed by using ImageJ software 1.53a (Java 1.8.0_172). (**b**) Immunofluorescence staining for Type I Collagen in human skin biopsies exposed to air pollutants and collected at the indicated timepoints. Green staining represents Type I Collagen, and the blue staining (DAPI) represents nuclei. Images were taken at 40× magnification and the fluorescent signal was quantified using ImageJ software 1.53a (Java 1.8.0_172). Data are the results of the averages of at least three different experiments, * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$ pollutants vs. Ctrl at the respective timepoints (DAY 1 and DAY 4) by 2-way ANOVA followed by Tukey's post-hoc comparison test.

2.3. Air Pollutants Differently Trigger NLRs Inflammasomes Activation in Human Skin Biopsies

Air pollutants have been demonstrated to induce the activation of inflammasomes, master regulators of the inflammatory response of many tissues, including skin [23,24]. Thus, the activation of inflammasomes was evaluated with the aim to understand whether there was a pollutant/NLRs inflammasome specific activation. The list of the air pollutants exerting an effect on inflammasomes components has been reported in Supplementary Figure S1.

First, it was evaluated the gene expression levels of the main *NLRs* inflammasomes such as *NLRP3*, *NLRP1*, *NLRC4* and *NLRP6* in human skin biopsies exposed to the different pollutants (MP, CS, DEE, O_3 and UV), for 1 (DAY 1) or 4 days (DAY 4). Results showed that beside *NLRC4* (Figure 3c), exposure to the different air pollutants could highly modulate NLRs inflammasomes in human skin. Indeed upon 4 days of exposure, human skin

biopsies showed a significant increase in *NLRP3* (Figure 3a), *NLRP1* (Figure 3b) and *NLRP6* (Figure 3d) genes expression compared to unexposed tissue. Among all air pollutants, UV displayed the strongest induction in almost all inflammasomes in response to a prolonged exposure (DAY 4). However, also MP, CS, DEE and O_3 induced a significant increase in *NLRP3, NLRP1* and *NLRP6* expression levels at DAY 4. *NLRP1* and *NLRP6* turned out to be particularly susceptible to CS and O_3 at day DAY 1 and upregulated by all air pollutants at DAY 4. MP displayed a tendency to affect *NLRs* inflammasomes induction upon a prolonged air pollutants exposure (DAY 4) rather than a short one (DAY 1). Although DEE exposure could modulate the expression of almost all *NLRs* genes, its effect did not show any tendency towards a specific inflammasome. *NLRC4* turned out to be the less modulated inflammasomes and was upregulated only by CS and UV after one day of exposure. Once activated inflammasomes receptor usually recruit the speck like receptor ASC to create a platform able to eventually promote the release of inflammatory cytokines including IL-1β [26]. Thus, we wondered whether air pollutants could promote the upregulation of ASC. As shown in Figure 3e, beside MP, at DAY 1 and O_3 at DAY 4, all air pollutants could promote an upregulation of ASC protein expression levels. Of note, UV seemed to have the strongest ability to modulate ASC expression in the short term (DAY 1) compared to the other pollutants, whereas MP, CS and DEE showed a prominent effect in ASC upregulation ad DAY 4.

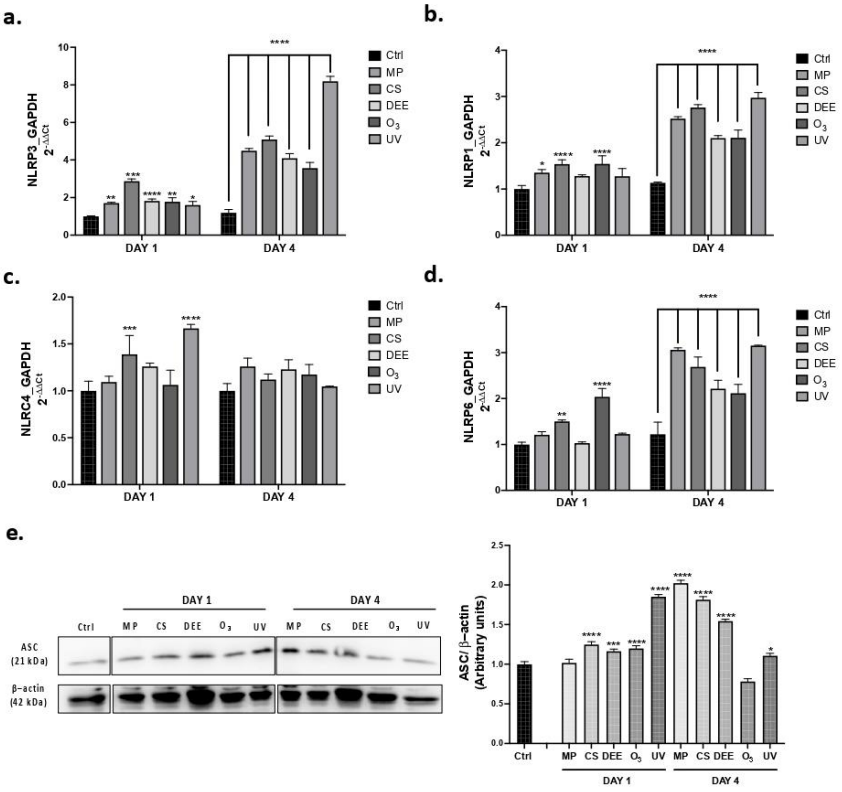

Figure 3. mRNA expression levels of NLRP3 (**a**), NLRP1 (**b**), NLRC4 (**c**) and NLRP6 (**d**) in human skin biopsies exposed to air pollutants and collected at DAY 1, DAY 2 and DAY 4 post exposure. (**e**) Protein

expression levels of ASC in human skin biopsies exposed to air pollutants and analyzed at the indicated timepoints. The protein expression level was quantified using ImageJ software 1.53a (Java 1.8.0_172) and β-actin was used as internal control. Data are the results of the averages of at least three different experiments, * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$ pollutants vs. Ctrl at the respective timepoints (DAY 1 and DAY 4) by 2-way ANOVA followed by Tukey's post-hoc comparison test.

2.4. NLRP1 Most Susceptible Cutaneous Inflammasome to Air Pollutants Exposure

To evaluate the inflammasomes scaffold formation upon air pollutants exposure, the colocalization levels between NLRs inflammasomes and ASC in human skin biopsies were assessed by IF staining. Among all inflammasomes measured, NLRP1 seemed to be the mostly modulated by air pollutants exposure in term of expression levels and colocalization with ASC, whereas NLRC4 turned out to be the less affected. As shown in Figure 4b, almost all air pollutants were able to upregulate NLRP1 expression levels in human skin biopsies at both timepoints, DAY 1 and DAY 4. Of note, the expression levels of ASC were also increased at each timepoint, followed by significant colocalization with NLRP1, especially after 4 days of exposure (DAY 4). These data suggest that NLRP1 might engage the speck-like receptor when activated. NLRP3 expression levels seemed to be upregulated only by DEE exposure after one day (DAY 1), whereas no significant changes in its expression levels were evident after 4 days (DAY 4) of exposure (Figure 4a). However, increased colocalization between NLRP3 and ASC occurred in response to MP and DEE exposure after 4 days (DAY 4).

The NLRC4 and ASC expression levels were upregulated only in response to UV at DAY 1. Exposure to the other air pollutants did not affect the NLRC4 expression levels and in some cases promoted a significant decrease in protein expression levels as well as in the colocalization with ASC (Figure 4c). Regarding NLRP6, its expression level increased after one day of exposure to MP, CS, and O_3 (DAY 1) and to O_3 upon 4 days of exposure (DAY 4) (Figure 4d). In this case, only O_3 exposure promoted an increase in ASC expression levels after 1 day (DAY 1) and 4 days (DAY 4) of exposure. UV, instead, seemed to downregulate NLRP6 expression as well as ASC, especially at DAY 1. Although a tendency towards colocalization between NLRP6 and ASC was visible upon all air pollutants' exposure, it was not significant compared to unexposed tissues at any timepoint, nor under any conditions.

2.5. Air Pollutant Exposure Induced the Activation of Cutaneous Inflammasome and Pyroptosis Responses

To evaluate the effective activation of the inflammatory pathways in response to the different air pollutants, the released levels of IL-1β in the media of the human skin biopsies were measured. As shown in Figure 5a, MP and O_3 clearly promoted an increase in IL-1β levels after 1 day of exposure (DAY 1), whereas MP, DEE, and UV seemed to induce IL-1β release mainly at longer timepoints (DAY 4) (Figure 5a). Inflammasome activation can eventually lead to a type of cell death named pyroptosis, which includes the formation of pores on the cell membrane that are able to promote the release of inflammatory mediators and propagate the inflammatory response within the tissue, resulting in cell death [34]. This process relies on the substrate Gasdermin D, whose cleavage in response to inflammasome activation generates a 30 kDa N-terminal fragment able to translocate to the cell membrane, where it can form pores [35]. Thus, to determine which air pollutants could eventually be correlated with this type of cell death, the expression levels of active p30 Gasdermin D over the pro-form p50 Gasdermin D in human skin biopsies was assessed by a Western blot. Results showed that after one day of exposure (DAY 1), MP, O_3, and UV were the main pollutants promoting the cleavage of Gasdermin D in human skin, whereas prolonged exposure resulted in the maturation of Gasdermin D in response to all air pollutants (Figure 5b). In this context, MP, CS, and UV seemed to exert the most evident effect.

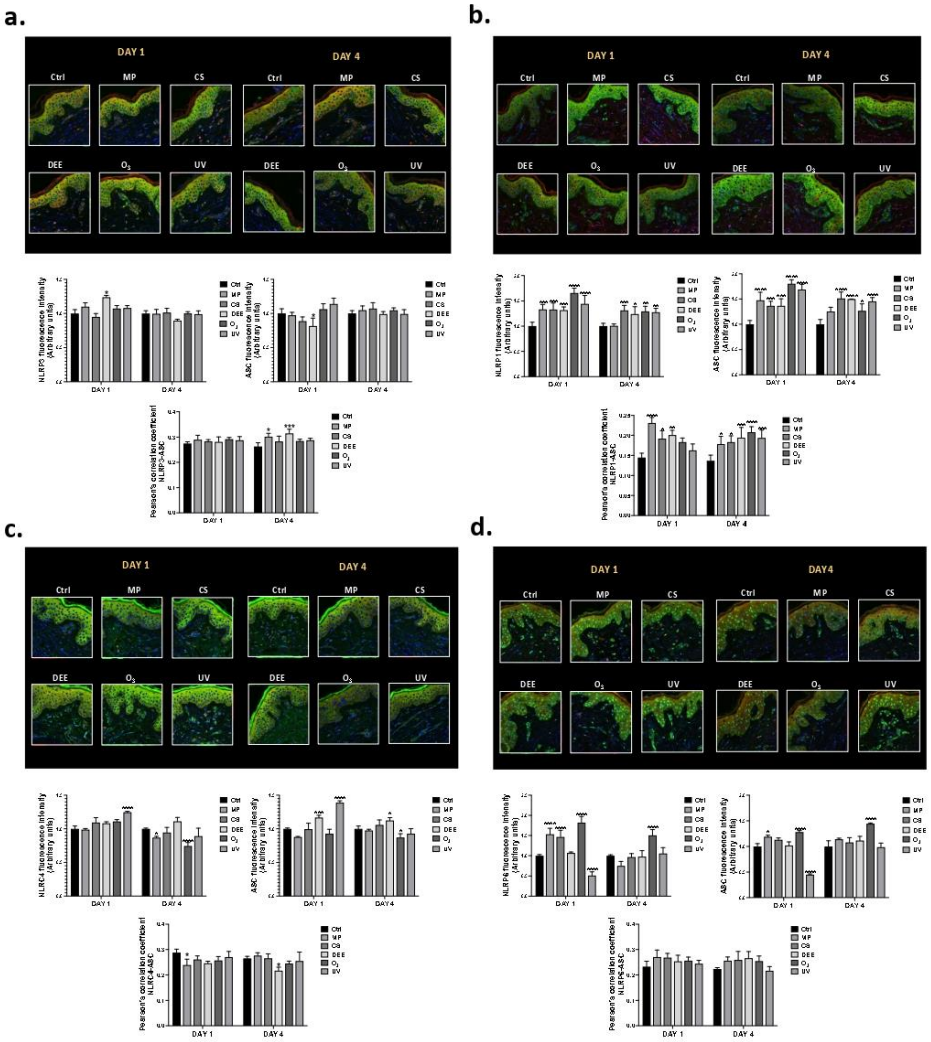

Figure 4. Immunofluorescence staining for NLRP3 (green staining) and ASC (red staining) (**a**); NLRP1 (red staining) and ASC (green staining) (**b**); NLRC4 (green staining) and ASC (red staining) (**c**); and NLRP6 (green staining) and ASC (red staining) (**d**) in human skin biopsies exposed to air pollutants and collected at the indicated timepoints. Blue staining (DAPI) represents nuclei. Images were taken at 40× magnification and the fluorescent signal was quantified using the ImageJ software 1.53a (Java 1.8.0_172). The colocalization between NLRP3, NLRP1, NLRC4, or NLRP6 and ASC is expressed as Pearson's correlation coefficient. Data are the results of the averages of at least three different experiments, * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$ pollutants vs. Ctrl at the respective timepoints (DAY 1 and DAY 4) by 2-way ANOVA followed by Tukey's post-hoc comparison test.

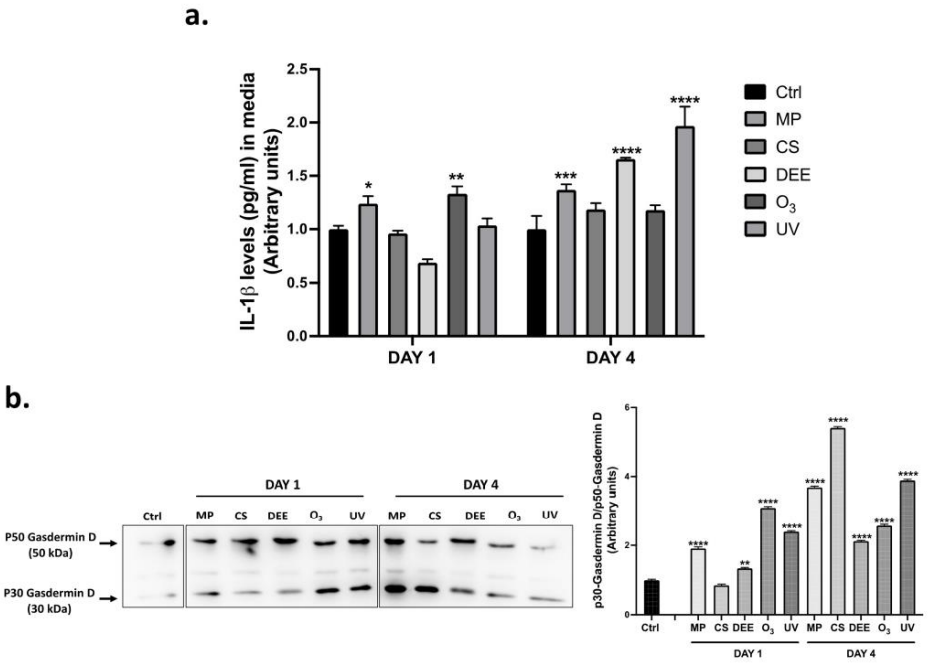

Figure 5. (**a**) Released levels of IL-1β expressed as pg/mL in media of human skin biopsies exposed to air pollutants and analyzed at DAY 1 and DAY 4. (**b**) Protein expression levels of p30 Gasdermin D over p50 Gasdermin D in human skin biopsies exposed to air pollutants and analyzed at the indicated timepoints. Protein expression level was quantified using the ImageJ software 1.53a (Java 1.8.0_172) and β-actin was used as an internal control. Data are the result of the averages of at least three different experiments, * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$ pollutants vs. Ctrl at the respective timepoints (DAY 1 and DAY 4) by 2-way ANOVA followed by Tukey's post-hoc comparison test.

3. Discussion

The exposure of human skin to environmental pollutants is nowadays recognized as one of the primary causes of the exacerbation of cutaneous disorders, cancer, and premature skin aging [2]. Air pollutants and UV have been shown to induce cutaneous extrinsic premature aging, which itself represents a response to an inflammatory and oxidative stress status (oxinflammation) [36].

For instance, environmental pollutants and UV were found to contribute to the exacerbation of skin photoaging and increased oxinflammatory reactions within the cutaneous tissues, which were counteracted by topical treatment with antioxidant compounds [37]. Clinical studies demonstrated the exacerbation of skin disorders and allergic reactions in subjects living in polluted areas. In Korea, children suffering from moderate to severe atopic dermatitis showed exacerbation of the pathology when exposed to indoor pollutants such as volatile organic compounds (VOCs) [38], and exposure to road traffic increased the allergic response in the skin of patients suffering from topic eczema/dermatitis compared to normal subjects [39]. In addition, PM2.5 exposure levels were positively associated with skin aging manifestation in the Chinese population [40].

The mechanism by which outdoor stressors affect target tissues, including the skin, is mainly through the alteration of the cellular redox homeostasis [41], leading to a cascade of oxidative stress reactions involving the depletion of skin surface antioxidants, cutaneous structural damage, and the impairment of barrier function [2]. All these events can culminate in the activation of inflammatory pathways, among which inflammasomes are now recognized as important modulators of the cutaneous inflammatory response to environmental insults [24].

Therefore, the aim of the present study was to understand how different air pollutants can promote the establishment of an oxidative and inflammatory environment. To do so, human skin biopsies were exposed for 1 or 4 days to different environmental agents such as MP, CS, DEE, O_3, and UV. Our results confirmed the presence of an oxidative stress environment in response to pollutant exposure, accompanied by increased DNA damage and the initiation of the skin aging process. We then focused our attention on the possible involvement of the main NLR inflammasomes (NLRP1, NLRP3, NLRP6, NLRC4) in the cutaneous response to environmental insults.

First, our results showed that exposure to air pollutants, particularly to MP and UV, promoted increased levels of 4HNE (Figure 1a), one of the most reactive products of lipid peroxidation. Since air pollutants can interact with the lipids present within the *stratum corneum*, leading to the production of ROS and consequent lipid peroxidation, 4HNE represents an important oxidative stress marker in skin conditions [15,42]. Moreover, 4HNE is involved in the regulation of several inflammatory and oxidative stress pathways, including the activation of redox-sensitive transcription factors such as NF-κB and NRF2. In addition, as an α-β unsaturated aldehyde, it is able to covalently react with proteins and form protein adducts (PAs), an event that can lead to changes in protein conformation and thereby in the alteration of their function, eventually resulting in cell death [43–48]. Many studies have shown the presence of higher 4HNE levels after O_3 exposure in several skin models, including in vitro, ex vivo (human explants), and in vivo (human and mouse models) [49]. Other pollutants, such as CS and PM, have also been associated with increased 4HNE PA levels in human skin and keratinocytes [9,50]. Along with the increased 4HNE levels, we also found altered expression of Sestrin 2 (SESN2) (Figure 1b), an evolutionarily conserved protein known to regulate the cellular antioxidant response and the aging process and play a role in tumor suppression, such as melanoma [51]. For example, the knockdown of *SESN2* in melanoma cells was followed by elevated oxidative stress levels and increased apoptosis rates, whereas the upregulation of the *SESN2* gene exerted a protective role by detoxifying oxidative stress species and disrupting subsequent melanoma formation [52]. Our results show that exposure to air pollutants can significantly decrease SESN2 levels even after one day (DAY 1) of exposure, and this effect was even more evident after 4 days of exposure (DAY 4), suggesting the compromised activity of the cellular defensive response. Air pollutants are indeed known for modulating the detoxifying responses of the skin, such as the aryl hydrocarbon receptor (AHR) and nuclear factor erythroid 2-related factor 2 (NRF2) transcription factors, which regulate cutaneous homeostasis [53]. Considering that NRF2 can bind to the antioxidant response element (ARE) within the SESN2 gene, promoting its expression to exert cytoprotective effects against oxidative stress [54,55], our data suggest that air pollutants may alter the antioxidant response of the cutaneous tissue, resulting in an inadequate tissue response to fight the increased oxidative damage.

Excessive ROS production can cause DNA damage via an indirect mechanism that leads to the formation of DNA oxidation products such as 8-OHdG [56]. All air pollutants induced an increase in 8-OHdG expression levels after one day and four days of exposure (Figure 1c). Among all air pollutants, the prolonged exposure to UV (DAY 4) seemed to particularly promote the oxidation of DNA, in line with the evidence that sun radiation represents the main cause of DNA mutation and skin cancer progression [57].

Upon ROS formation, skin cells can release a variety of pro-inflammatory mediators, resulting in an inflammatory status with activated neutrophils and immune cell infiltration, which can generate further free radicals, establishing a vicious cycle named

oxinflammation [3]. The continuous secretion of pro-inflammatory mediators (e.g., cytokines, chemokines) can promote the degradation of the connective tissue of the dermis by inducing the activation of matrix metalloproteinases (MMPs). These enzymes can target the degradation of structural proteins of the extracellular matrix (ECM) as different types of collagens, elastins, etc., ultimately affecting the dermis and its functionality and the formation of wrinkles (skin aging). Besides UV, exposure to various air pollutants can also contribute to this process by inducing the redox activation of MMPs, resulting in ECM matrix component degradation [36]. For instance, PM, one of the main components of DEE, has been shown to promote the induction of several MMPs, such as MMP-1, MMP-2, and MMP-9, in human keratinocytes via the ROS/JNK pathway, and treatment with natural antioxidant compounds such as hesperidin was able to partially prevent PM-induced skin damage [58]. Hairless mice exposed to O_3 displayed increased levels of 4HNE, accompanied by an increase in MMP-9 mRNA and activity levels [59]. Similarly, our results indicated that the prolonged exposure to air pollutants promoted an increase in active MMP-2 in skin biopsies (Figure 2a), especially in response to UV. In line with this result, pro-inflammatory markers as well as the induction of proteolytic enzymes such as MMPs in response to UV have been shown to favor the formation of a microenvironment more suitable for tumor progression and metastasis [60,61]. In parallel to MMP-2 induction, we also found decreased levels of type I collagen, especially at DAY 4, where DEE and UV represented the most dangerous insults (Figure 2b). It is worth mentioning that among all the environmental insults to which the skin biopsies were exposed, MP was found to be one of the major air pollutants triggering oxidative stress (4HNE, SESN2), DNA damage (8-OHdG), and skin aging (MMP2, type I collagen). Considering that the major entry routs for MP are the digestive and respiratory systems, the direct impact that MP can exert on human skin is still poorly understood and under investigation. The presence of the *stratum corneum* in the outermost layer of the epidermis makes cutaneous MP absorption difficult, resulting in MP penetration mainly when the skin is damaged and it is easier to pass through (through wounds, hair follicles, etc.). Our results demonstrate, for the first time, that MP has a direct impact on human skin by altering skin redox homeostasis and even promoting DNA damage. Apart from the direct damage that MP can exert while interacting with human skin, it is possible to hypothesize that inhaled or ingested MP can reach the skin from the bloodstream, making this relatively "new" pollutant one of the most toxic to the cutaneous tissue.

The activation of the inflammatory pathways known as inflammasomes has garnered immense attention in the past few decades in terms of mediating the inflammatory response related to cutaneous disorders [24]. For instance, genetic variations of the NLRP1 and NLRP3 inflammasomes have been associated with high levels of IL-1β in the lesions of patients suffering from psoriasis, vitiligo, and atopic dermatitis [30,32,62–70]. Increasing evidence in the past few decades has shown that air pollutants can trigger inflammasome activation in human skin via ROS production [24]. As an example, NLRP1 function was found to be compromised in human keratinocytes upon PM exposure via the ROS/NFkB pathway. Moreover, numerous pieces of evidence correlate UV exposure with the activation of cutaneous inflammasomes, especially via DNA damage and ROS production and subsequent IL-1β and IL-18 release [24,71]. Exposure to O_3 was found to promote cutaneous NLRP1 inflammasome activation via oxidative stress mediators such as 4HNE and H_2O_2; similarly, CS was found to differently modulate the NLRP3 and NLRP1 inflammasomes in human keratinocytes [72]. Although NLRP1 and NLRP3 represent the most studied inflammasomes in human skin, there is some evidence suggesting that cutaneous NLRC4 can be activated by UV [73].

While the number of studies correlating the exposure of air pollutants to cutaneous inflammasome activation has tremendously increased in the past few years, limited information is available on how the same inflammasome receptor can differently respond to specific pollutants.

In the present study, we found that NLRP1 may represent the major inflammasome involved in the cutaneous inflammatory response against environmental insult. The results showed that the *NLRP3* mRNA expression levels were upregulated by all air pollutants, whereas *NLRP1* and *NLRP6* were particularly susceptible to O_3 and CS at DAY 1 and to all air pollutants upon prolonged exposure (DAY 4) (Figure 3a–d). The *NLRC4* inflammasome was the least upregulated by the environmental insults, only responding to UV (Figure 3c). Moreover, among all the inflammasomes, only NLRP1 seemed to colocalize with ASC, already at DAY 1 but more evidently at DAY 4, suggesting that NLRP1 may represent the inflammasome that is most involved in the cutaneous inflammatory response triggered by environmental insults (Figure 4b). These data are in alignment with the evidence that NLRP1 is the most expressed NLR receptor in human skin [74]. Following NLRP1, NLRP6 also seemed to be particularly susceptible to air pollutant exposure. Indeed, although the colocalization with ASC did not occur properly, the air pollutants increased the NLRP6 expression levels both at DAY 1 and DAY 4 (Figure 4d). Regarding the other inflammasomes, NLRC4 seemed to be the least modulated one in human skin (Figure 4c), confirming the mRNA expression level results. However, in accordance with other studies conducted on NLRC4, UV was found to be the only environmental stimulus able to modulate the NLRC4 inflammasome both at the gene and protein expression levels [73].

Although we found that NLRP3 was the major inflammasome modulated at the gene expression level, the immunofluorescence analysis demonstrated that this inflammasome could be partially activated by some of the air pollutants with lower co-localization with ASC compared to the other NLR inflammasomes (Figure 4a). This could be explained via the hypothesis that NLRP3 activation in response to air pollutants might be a very fast event, faster than the earliest timepoint analyzed in our study; on the other hand, under noxious stimuli, the cutaneous NLRP3 inflammasome might be subjected to modifications occurring at the post-translational level that affect its activity. Post-translational modification (PTM) events, including ubiquitination, SUMOylation, phosphorylation, etc., can modulate inflammasome activation in many contexts, including in response to air pollutants, resulting in their activation or inhibition [75]. For example, CS exposure can promote NLRP3 inhibition via ubiquitination [76], whereas O_3 can promote NLRP1 activation via the ubiquitin-proteasome system. Moreover, it is worth mentioning that although colocalization with ASC rarely occurred for almost all the NLR receptors under our experimental conditions, the role of ASC in inflammasome activation is still not completely clear, since it is not always required for the inflammasomes' activation. For instance, there is evidence demonstrating that since NLRC4 does not display a PYD domain, it can directly interact with caspase 1 without recruiting ASC [77]. NLRP1 can also directly interact with caspase 1 due to the presence of an additional CARD domain in its C-terminus that can directly recruit pro-caspase 1 [78], as well as another inflammatory caspase, caspase 5 [79]. Considering that ASC is predominantly colocalized with NLRP1, it is plausible that the significant increase in the total ASC protein expression levels found in the human skin biopsies in response to air pollutant exposure (Figure 3e) might have been due to NLRP1 activation. Nevertheless, our data showed that, besides NLRP1, NLRP6 was the preferred inflammasome modulated by the environmental pollutants in the skin biopsies. This was the most surprising result, considering that, while NLRP1, NLRP3, and NLRC4 are the most studied inflammasomes in human skin, evidence surrounding the role of NLRP6 in the cutaneous inflammatory response is scarce. NLRP6 is predominantly expressed in the gastrointestinal tract, where it regulates the inflammatory response and the intestinal microbiota in response to infectious stimuli. Interestingly, it has been shown that NLRP6 can upregulate the antimicrobial peptides (AMPs) naturally present in the gut microbiome, protecting it from infectious bacteria [80,81]. Considering that human skin is equipped with AMPs that act as antimicrobial agents, which also regulate the activation of immune cells and the production of pro-inflammatory cytokines [82,83], it is plausible that NLRP6 could modulate cutaneous AMPs in the regulation of the inflammatory response as well. Indeed, a range of evidence shows a strict link between the gut and skin (gut–skin axis),

demonstrating an interconnection between the intestinal and cutaneous microbiomes in the manifestation of skin conditions [84]. Of note, increased levels of cutaneous AMPs have been detected in many skin diseases, such as atopic dermatitis and psoriasis [85,86]. Moreover, exposure to O_3 has been shown to upregulate cutaneous AMPs such as human betadefensins (HBDs) and cathelicidin LL-37 [87], supporting the fact that, in this study, O_3 showed the greatest effect on NLRP6 modulation both at the gene and protein expression levels. The activation of inflammasomes by pollutant exposure was finally confirmed by the release of the cytokine IL-1β in the media of the human skin biopsies, along with upregulated levels of the pyroptosis mediator Gasdermin D (Figure 5a,b). MP and O_3 led to the complete activation of the inflammatory pathways after the first period of exposure, as depicted by the increased released levels of IL-1β found in the media of the human skin biopsies, whereas DEE and UV seemed to induce inflammasome activation more in the long term (DAY 4). In addition, exposure to air pollutants promoted the activation of the pyroptosis effector p30 Gasdermin D, whose levels were upregulated both in the short and long term (Figure 5b). Considering that the release of IL-1β found in the media of skin biopsies after the first period of exposure seemed to be particularly high in response to MP and O_3, as well as the levels of mature Gasdermin D, it is possible that MP and O_3 represent the main pollutants promoting pyroptosis as an acute response in human skin. Moreover, our results suggest that NLRP1, followed by NLRP6, may represent the preferred inflammasome receptors involved in the MP- and O_3-induced cutaneous inflammatory response in the short term (DAY 1).

In conclusion, our study shows the different effects that air pollutants may exert on the markers of the oxidative and inflammatory responses and the skin aging process. In particular, as summarized in Figure S1, O_3 appeared to represent the air pollutant that was most likely to affect inflammasome activation in human skin as an acute response (DAY 1), whereas exposure to UV was found to be the preferred environmental insult able to activate the inflammasomes in the long term (DAY 4). Since UV is also responsible for inducing the greatest damage in terms of DNA oxidation and the activation of MMP-2, it is plausible that the inflammasomes are involved in the establishment of the inflammatory status, contributing to the skin aging process (inflammaging) [25]. Interestingly, among all air pollutants, MP displayed the highest impact in terms of oxidative stress marker induction, such as 4HNE and SESN2, in the short term (DAY 1), along with a tendency to promote an inflammatory status, as displayed by increased levels of IL-1β and the activation of Gasdermin D.

We believe that this is the first study showing the ability of MP to affect the skin's physiology and it may represent an important modulator of the inflammasome response in human skin. Considering that daily, concomitant exposure to pollutants can synergistically affect the cutaneous oxinflammatory status and compromise the skin barrier function [88], it is plausible that MP can not only exacerbate the air pollutants' cutaneous damage, but they may more easily penetrate within the *stratum corneum*, thus becoming important noxious stimuli for the skin. For instance, MP exposed to UV were found to be subjected to oxidation or be more prone to release by plastic films, thus becoming even more dangerous to the body [19].

In addition, we also showed for the first time that NLRP6, along with NLRP1, was particularly susceptible to the effect of air pollutants in human skin, thus becoming an important molecular target involved in the inflammatory response of the cutaneous tissue.

4. Materials and Methods

4.1. Skin Biopsies, Culture, and Treatments

Human skin explants were obtained from elective abdominoplasties and the protocol was approved by the Institutional Biosafety (IBC) Committee at NC State University. Subcutaneous fat was removed from the abdominoplasties and 12 mm punch biopsies were collected and rinsed in PBS. Biopsies were then placed in 6-well plates and cultured in DMEM high-glucose containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL

streptomycin at 37 °C in 5% CO_2. After 24 h of recovery in the incubator, the culture medium was replaced with fresh medium, and the biopsies were exposed to the different air pollutants [88]. Pollutant exposure was performed as follows: 20 µL of a 500 µg/mL microplastics solution (cat. 102 Phosphorex, LLC, Hopkinton, MA, USA, 01748) was first applied to skin biopsies using a glass rod. Skin biopsies were incubated for 24 h and then exposed to 200 mJ UVA/UVB light [88] or 0.25 ppm of O_3 for 2 h as previously described, 30 min of diesel engine exhaust (DEE) as in reference [89], or 30 min of cigarette smoke. Skin biopsies were exposed every day for 4 days and the samples were collected 24 h after the end of the first air pollutant exposure (DAY 1) or after 4 days (DAY 4). Medium was changed every day before exposure to air pollutants and collected at the indicated timepoints for further analysis.

4.2. Immunofluorescence Staining

Human skin samples were fixed in 10% neutral buffered formalin for 24 h RT and dehydrated in increasing concentrations of alcohol (70, 80, 90, and 100%) for 30 min and then in xylene for 45 min, twice, using an automated Leica tissue processor (Leica Biosystems, Deer Park, IL, USA). The samples were left in paraffin overnight and embedded in paraffin to create blocks. For immunohistochemical analysis, 4 µm sections of skin biopsies were cut using a microtome (Leica Biosystems, Deer Park, IL, USA) and then deparaffinized in xylene and rehydrated in a decreasing alcohol gradient as previously described [88]. The sections were subjected to heat-based antigen retrieval using a sodium citrate buffer (C9999, Sigma-Aldrich, Merck Millipore, Burlington, MA, USA) (pH 6.0) at a sub-boiling temperature of 95 °C in a water bath for 10 min. Samples were cooled down to room temperature for 25 min, washed twice in PBS and then blocked with 5% BSA in PBS for 45 min at room temperature. Sections were then incubated overnight with primary antibodies at 4 °C as follows: NLRP1 (sc-166368Santa Cruz Biotechnology Inc., Dallas, TX, USA), NLRP3 (cat. NBP1-97601, Novus Biological, Littleton, CO, USA) 1:100, NLRP6 (NBP2-31372, Novus Biological, Littleton, CO, USA) 1:200, NLRC4 (NB100-561425, Novus Biological, Littleton, CO, USA) 1:200, ASC (cat. NBP1-78977, Novus Biological, Littleton, CO, USA) 1:150 or ASC (sc-271054, Santa Cruz Biotechnology Inc., Dallas, TX, USA) 1:50, 8-OHdG (sc-393871, Santa Cruz Biotechnology Inc., Dallas, TX, USA), type I collagen (ab-138492, abcam, Cambridge, UK), Sestrin 2 (cat. 10795-1-AP, Proteintech, Rosemont, IL, USA), 4HNE (AB5605, MilliporeSigma, Burlington, MA, USA) PBS, BSA 0.25%. The day after, samples were washed in PBS 3 times for 5 min and incubated with fluorochrome-conjugated secondary antibodies (dil. 1:1000) (Alexa Fluor 568 A11004 or Alexa Fluor 488 A11055) in 0.25% BSA in PBS at RT. Nuclei were stained with DAPI (D1306 Invitrogen, Thermo-Fisher Scientific, Waltham, MA, USA). Coverslips were mounted onto glass slides using Fluoromount-G™ Mounting Medium (00-4958-02, ThermoFisher Scientific, Waltham, MA, USA), and examined using a Zeiss Z1 AxioObserver LSM10 confocal microscope at 40× magnification. Images were quantified using the ImageJ software 1.53a (Java 1.8.0_172, National Institutes of Health, Bethesda, MD, USA).

4.3. RNA Extraction and Quantitative Real-Time PCR (rt-PCR)

RNA extraction from human skin biopsies was performed using TRIzol™ Reagent (Cat. 15596026, Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) as previously described in [87]. Briefly, skin samples were placed in Precellys Eppendorf tubes containing metallic beads (cat. 15-340-151, Fisherbrand, ThermoFisher Scientific, Waltham, MA, USA) and 700 µL of Trizol was added to the samples. Tissues were then homogenized using a TissueLyser (Qiagen, Germantown, MD, USA) equipped with liquid nitrogen, by performing 3 cycles of 6500 rpm for 30 s each. Samples were then subjected to a modified version of the phenol–chloroform extraction protocol described previously [6]. Briefly, 140 µL RNase-free chloroform was added to each tube and then centrifuged for 15 min at 12,000× *g* at 4 °C. The aqueous phase containing RNA was transferred to a new tube and the chloroform step was repeated. RNA precipitations were assessed by adding 350 µL of

RNase-free isopropanol and centrifuging at 4 °C, 12,000× *g* for 10 min. The RNA pellet was washed 3 times in 75% EtOH by centrifuging the samples at 4 °C, 10,000× *g* for 10 min. The pellet was then suspended in 35 µL of nuclease-free water and quantified using a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Then, 1 µg of total RNA from each sample was reverse transcribed in cDNA using the iScript cDNA Synthesis Kit (cat. 1708841, Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's instructions. The mRNA levels of NLRP1, NLRP3, NLRP6, ASC, and IL-1β were analyzed by performing a quantitative real-time PCR using the SsoAdvanced Universal SYBR Green Supermix (cat. 1725271, Bio-Rad Laboratories, Inc., Hercules, CA, USA), on a Roche LightCycler 480 machine, according to the manufacturer's protocol.

Gene expression was quantified based on the number of cycles necessary to reach a predetermined threshold value (Ct value) for each sample, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was employed as the reference gene. After normalization, the fold change was determined using the $2^{-\Delta\Delta CT}$ method. The primer sequences are listed in the Table 1.

Table 1. Primers sequences.

Gene	Forward Sequence	Reverse Sequence
NLRP1	ACCCTCTTAACTCCGGGACA	GAGTGCGCTTTATTGGCGAG
NLRP3	CGGGGCCTCTTTTCAGTTCT	CCCCAACCACAATCTCCGAA
NLRP6	CCTGTGAAGGAATCACCTCTCT	GTCCATGGGGTCTCTTCCTCC
ASC	ATGCGCTGGAGAACCTGA	TCTCCAGGTAGAAGCTGACCA
IL-1β	CACGATGCACCTGTACGATCA	GTTGCTCCATATCCTGTCCCT
GAPDH	TCGGAGTCAACGGATTTGGT	TTCCCGTTCTCAGCCTTGAC

4.4. Protein Extraction and Western Blotting

Skin explants were placed in Precellys Eppendorf tubes containing metallic beads (cat. 15-340-151, Fisherbrand, ThermoFisher Scientific, Waltham, MA, USA) and homogenized as previously described [88]. Briefly, 350 µL of T-PER™ Tissue Protein Extraction Reagent (cat. 78510, ThermoFisher Scientific, Waltham, MA, USA) containing Halt™ Protease Inhibitor Cocktail (100×) (cat. 78429 ThermoFisher Scientific, Waltham, MA, USA) was added to each sample and tissues were subjected to 3 cycles of 6500 rpm, 30 s each, using the TissueLyser (Qiagen, Germantown, MD, USA) equipped with liquid nitrogen. Samples were then centrifuged at 16,000× *g* rpm, 30 min at 4 °C, and the protein content was then measured using Bradford assays and equivalent amounts of proteins were loaded onto polyacrylamide SDS gels. Gels were electroblotted onto nitrocellulose membranes (0.45 µm pore size) (cat. 1620115, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked in PBS-Tween 0.5% with 5% non-fat dry milk. The following primary antibodies diluted in PBS-T and milk 1% were used to incubate the membranes overnight at 4 °C: ASC (cat. NBP1-78977, Novus Biological, Littleton, CO, USA) 1:1000 and Gasdermin D (sc-393581, Santa Cruz Biotechnology Inc., Dallas, TX, USA) 1:500.

The day after, the membranes were washed in PBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (cat. 170–6515, 170–6516, Bio-Rad Laboratories, Inc., Hercules, CA, USA) diluted 1:5,000 in TBS-T with 1% non-fat milk for 90 min at RT. Bound antibodies were detected by chemiluminescence via the Clarity Western ECL Substrate (cat. 1705061, Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). β-actin (A3854 Sigma-Aldrich, Burlington, VT, USA) was used as a loading control at a 1:50,000 dilution in TBS-T with 1% non-fat milk. Densitometry analysis was performed using the Image J software 1.53a (Java 1.8.0_172, National Institutes of Health, Bethesda, MD, USA).

4.5. MMP-2 Zymography

The protein content of media samples was quantified using Bradford assays. First, 10 µg of the media sample was loaded into an 8% acrylamide 1 mm gel and run at 125 V for 90 min. Gels were carefully removed and placed in washing buffer (2.5% Triton X-100, 50 mM Tris HCl, 5 mM $CaCl_2$, H_2O) for 30 min at room temperature with gentle agitation twice. The washing buffer was then decanted and rinsed with 100 mL of double-distilled water. The gel was incubated for 30 min in 100 mL of developing buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 200 mM NaCl, H_2O) with gentle agitation; the solution was then decanted and replaced with fresh developing buffer overnight at room temperature. The next day, the developing buffer was removed, and the gel was stained with 50 mL of staining solution (0.1% Coomassie Blue) for 1 h with agitation. Following staining, the gel was washed with double-distilled water until excess staining solution was removed. The gels were then washed with destaining solution (50% H_2O, 40% methanol, 10% acetic acid) until gelatinolytic activity was evident. Images were then taken with the ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and bands were quantified using the ImageJ software 1.53a (Java 1.8.0_172, National Institutes of Health, Bethesda, MD, USA).

4.6. ELISA for IL-1β

IL-1β levels were measured in the media of skin biopsies collected at the indicated timepoints using the IL-1β ELISA kit (cat. DY201-05, Novus Biologicals, Centennial, CO 80112, USA), according to the manufacturer's instructions. The absorbance was measured with a spectrophotometer equipped with a filter of 450 nm, using 570 nm as a reference wavelength. IL-1β levels were expressed as pg/mL in culture media according to the manufacturer's instructions, and the Gen5 2.0 software (BioTek, Agilent, Santa Clara, CA, USA) was used for the detection.

4.7. Statistical Analysis

The statistical analysis was performed using GraphPad Prism 9 (Version 9.4.1 (458), GraphPad Software Inc., La Jolla, CA, USA) with an analysis of variance (1-way or 2-way ANOVA), followed by Tukey's post-hoc test, for each of the variables tested. Data are expressed as the mean ± SD of duplicate determinations obtained in three independent experiments, and statistical significance was considered at $p < 0.05$. For all experiments, control values were set to 1.0 and other values expressed as a fold change.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms242316674/s1.

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