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CICLO XXI

GENETIC VARIANTS C242T and -930 A/G OF THE *p22 phox* NAD(P)H OXIDASE POLYMORPHISM and VASODILATATION ENDOTHELIUM-DEPENDENT IN ESSENTIAL HYPERTENSION

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RIASSUNTO

Fattori genetici e stress ossidativo alterano la funzione endoteliale (E) ed è stato ipotizzato che varianti dei geni che codificano le subunità della nicotinamina adenina dinucleotide fosfato (NADPH) ossidasi, fonte principale di radicali liberi dell'ossigeno (ROS), siano implicate nella patogenesi della disfunzione endoteliale (DE). Quest'ultima rappresenta un connotato dell'ipertensione arteriosa essenziale ed è ritenuta svolgere un ruolo cruciale in tutte le fasi dell'aterogenesi.

La *p22phox* umana è una delle subunità della NAD(P)H ossidasi, è codificata dal gene CYBA, collocato sul braccio lungo del cromosoma 16 in posizione 24, grande 7,75 kb e si compone di sei esoni e cinque introni che codificano per un open reading frame (ORF) di circa 600 bp.

Sono noti numerosi SNPs della p22phox, ma quelli da noi esaminati sono stati:

- il   -930 A/G che si trova nella regione del promotore, alla posizione -930 dal codone ATG. Questo polimorfismo si trova su un sito potenzialmente vincolante per il fattore di trascrizione C/EBP (CCAAT/enhancer-binding protein);
- il C242T collocato sull'esone4.

Lo scopo del lavoro è stato quello di valutare se ai due single nucleotide polymorphism (SNPs), -930 A/G e C242 del gene p22phox (NC_000016.8), corrisponde una diversa produzione di anione superossido ($O_2^{\circ}-$) coinvolto nello stress ossidativo e valutare se i polimorfismi suddetti modificano l'emivita dell'enzima stesso.

E' stato valutato il ruolo di questi due SNPs in quindici pazienti, genotipizzati per entrambi i polimorfismi ed è stato condotto uno studio sulle cellule endoteliali estratte dalle vene giugulari di questi pazienti.

E' risultato che la mutazione sul promotore può realmente cambiare la capacità di legame del fattore di trascrizione C/EBP, modulando in maniera negativa l'attività trascrizionale di CYBA e la mutazione sull'esone 4 potrebbe ridurre lo stress ossidativo.

Questi risultati aiuterebbero l'identificazione di pazienti a elevato rischio di malattie cardiovascolari (CAD), al di là dei classici fattori di rischio già conosciuti.

SUMMARY

Genetic factors and oxidative stress affect endothelial (E) function and has been hypothesized that variants of genes that encode subunit of nicotinamide adenine dinucleotide phosphate oxidase complex (NADPH oxidase), a major source of reactive oxygen species (ROS) are implicated in the pathogenesis of endothelial dysfunction (ED). The latter is a feature of essential hypertension and is considered to play a crucial role in all phases of   atherogenesis.

The human *p22phox* is a subunit of NAD (P) H oxidase, encoded by the gene CYBA, located on the long arm of chromosome 16 in position 24, a large 7.75 kb and consists of six exons and five introns that encode for an open   reading   frame   (ORF)   of   600   bp. There   are   many   SNPs   of   p22phox,   but   we   have   investigated:

• -930 A/G, located in the promoter region at position -930 from the ATG codon. This polymorphism is located on a potential binding site for CCAAT/enhancer-binding   the   transcription   factor C/EBP (CCAAT/enhancer-binding protein);

• C242T on the exon 4.

The aim was to assess whether the two single nucleotide polymorphism (SNPs), -930 A/G and C242 of the p22phox gene (NC_000016.8), corresponds to a different production of superoxide anion ($O2°-$) involved in the stress oxidative and assess whether these polymorphisms modify the half-life   of   the   enzyme   itself. Examined the role of these two SNPs in fifteen patients, genotyped for both polymorphisms and was conducted a study on endothelial cells extracted from the jugular veins of these patients. Showed that the mutation on the promoter can really change the binding capacity of the transcription factor

C/EBP by modulating negatively the transcriptional activity of CYBA and mutation of the exon 4 could reduce oxidative stress. These results could help the identification of patients at high risk of cardiovascular disease (CAD), beyond the classic risk factors already known.

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Abbreviations:

NAD(P)Hox= NADH/NAD(P)H oxidase

ACH = Acetylcholine

BP= blood pressure

CV= cardiovascular

ED= Endothelial dysfunction

EDV= Endothelium-Dependent vasodilatation

EIV= Endothelium-Independent vasodilatation

FBF = Forearm Blood Flow

FRET = Fluorescence Resonance Energy Transfer

NO = Nitric Oxide

$(O_2^{\circ-})$ = superoxide anion

PCR= polymerase chain reaction

PH = Primary (essential) hypertension

SNP =single nucleotide polymorphism

SP = Sodium nitroprusside

1. CARDIOVASCULARE DISEASE

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels and include: coronary heart disease (disease of the blood vessels supplying the heart muscle), cerebrovascular disease (disease of the blood vessels supplying the brain), peripheral arterial disease (disease of blood vessels supplying the arms and legs), rheumatic heart disease (damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria), congenital heart disease (malformations of heart structure existing at birth), deep vein thrombosis and pulmonary embolism (blood clots in the leg veins, which can dislodge and move to the heart and lungs).

The last update (2009) of American Heart Association (AHA) showed that an estimated 80.000.000 American adults (one in three) have one or more types of cardiovascular disease (CVD), of whom 38.100.000 are estimated to be age 60 or older. The estimates were extrapolated to the U.S. population in 2006 from NHANES 2005–06 data.

The update showed, furthermore, that 73.600.000 american patients has High blood pressure (HBP) — (defined as systolic pressure 140 mmHg or greater and/or diastolic pressure 90 mmHg or greater, taking antihypertensive medication or being told at least twice by a physician or other health professional that you have HBP), 16.800.000 has Coronary heart disease (CHD) of this:

- Myocardial infarction (MI, or heart attack) — 7.900.000

- Angina pectoris (AP, or chest pain) — 9.800.000,

5.700.000 has Heart failure (HF), 6.500.000 Stroke and 650.000 – 1.300.000 Congenital cardiovascular defects.

The following prevalence estimates are for people age 18 and older from NCHS NHIS, 2007.

The incidence is based on the NHLBI's Framingham Heart Study (FHS) original and offspring cohort (1980–2003):

– the average annual rates of first major cardiovascular events rise from three per 1.000 men at ages 35–44 to 74 per 1.000 at ages 85–94. For women, comparable rates occur 10 years later in life. The gap narrows with advancing age.

– before age 75, a higher proportion of CVD events due to CHD occur in men than in women, and a higher proportion of events due to congestive heart failure (CHF) occur in women than in men.

Data from the FHS indicate that the lifetime risk for CVD is two in three for men and more than one in two for women at age 40. Final mortality data show that CVD as the underlying cause of death (including congenital cardiovascular defects) accounted for 35.3 percent (864.480) of all 2.448.017 deaths in 2005, or one of every 2.8 deaths in the United States. CVD total mention deaths (1.372.000 deaths in 2005) accounted for about 56 percent of all deaths in 2005.

In every year since 1900, except 1918, CVD accounted for more deaths than any other single cause or group of causes of death in the United States. Nearly 2.400 Americans die of CVD each day, an average of one death every 37 seconds. CVD claims about as many lives each year as cancer, chronic lower respiratory diseases, accidents and diabetes mellitus combined. The 2005 overall death rate from CVD was 278.9. The rates were 324.7 for white males and 438.4 for black males; 230.4 for white females and 319.7 for black females. From 1995–2005, death rates from CVD declined

26.4 percent. In the same 10-year period, actual CVD deaths declined 9.6 percent. Other causes of death in 2005 cancer, 559.312; accidents, 117.809; Alzheimer's disease, 71.599; HIV (AIDS), 12.543. Final 2005 CVD death rates were 331.1 for males and 237.1 for females. Cancer (malignant neoplasms) death rates were 225.1 for males and 155.6 for females. Breast cancer claimed the lives of 41.116 females in 2005; lung cancer claimed 69.105. Death rates for females were 24.1 for breast cancer and 40.5 for lung cancer. One in 30 female deaths was from breast cancer, while one in six was from CHD. By comparison, one in 4.6 female deaths was of cancer while one in 2.7 was of CVD. Based on 2005 mortality, CVD caused about a death a minute among females — about 455.000 female lives in 2005. That's more female lives than were claimed by cancer, chronic lower respiratory diseases, Alzheimer's disease, accidents and diabetes combined. Nearly 151.000 Americans killed by CVD (I00-I99) in 2005 were under age 65. In 2005, 32 percent of deaths from CVD occurred prematurely (i.e., before age 75, which is well below the average life expectancy of 77.8 years).

According to the NCHS, if all forms of major CVD were eliminated, life expectancy would rise by almost seven years. If all forms of cancer were eliminated, the gain would be three years. According to the same study, the probability at birth of eventually dying from major CVD is 47 percent and the chance of dying from cancer is 22 percent. Additional probabilities are 3 percent for accidents, 2 percent for diabetes and 0.7 percent for HIV.
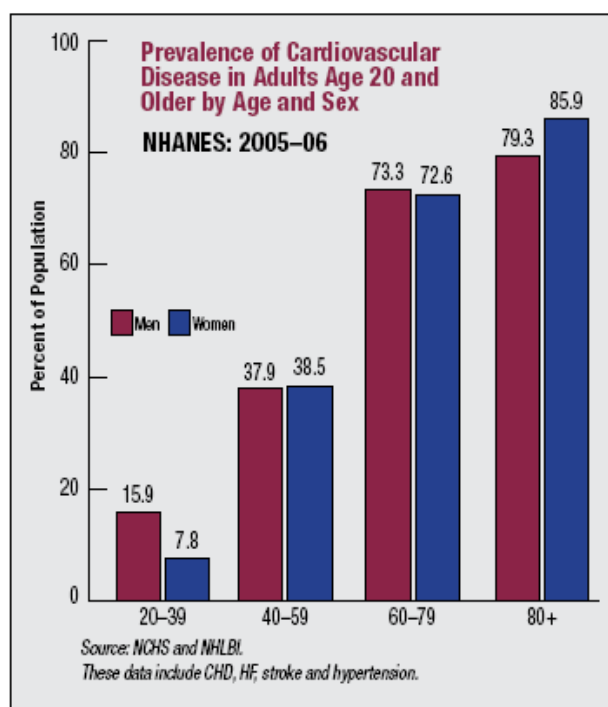
Fig.1 Prevalence of the Cardiovascular Disease in Adults

What are the risk factors for cardiovascular disease?

The most important behavioural risk factors of heart disease and stroke are unhealthy diet, physical inactivity and tobacco use. Behavioural risk factors are responsible for about 80% of coronary heart disease and cerebrovascular disease. The effects of unhealthy diet and physical inactivity may show up in individuals as raised blood pressure, raised blood glucose, raised blood lipids, and overweight and obesity; these are called 'intermediate risk factors'. There are also a number of underlying determinants of CVDs, or, if you like, "the causes of the causes". These are a reflection of the major forces driving social, economic and cultural change – globalization, urbanization, and population ageing. Other determinants of CVDs are poverty and stress.

In according to the World Healt organization (WHO), the hypertension (HT) represents a most risk factor in the CV disease, because has the highest prevalence.

2. HYPERTENSION INDUCED END-ORGAN DAMAGE

Hypertension is a chronic medical condition in which the blood pressure is elevated. It is also referred to as high blood pressure or shortened to HT, HTN or HPN. The word "hypertension", by itself, normally refers to systemic, arterial hypertension.[41]

Hypertension can be classified as either essential (primary) or secondary. Essential or primary hypertension means that no medical cause can be found to explain the raised blood pressure. It is common. About 90-95% of hypertension is essential hypertension.[42,43,44] Secondary hypertension indicates that the high blood pressure is a result of (*i.e.,* secondary to) another condition, such as kidney disease or tumors (adrenal adenoma or pheochromocytoma).

Persistent hypertension is one of the risk factors for strokes, heart attacks, heart failure and arterial aneurysm, and is a leading cause of chronic renal failure[45]. Even moderate elevation of arterial blood pressure leads to shortened life expectancy. At severely high pressures, defined as mean arterial pressures 50% or more above average, a person can expect to live no more than a few years unless appropriately treated[46]. Beginning at a systolic pressure (which is peak pressure in the arteries, which occurs near the end of the cardiac cycle when the ventricles are contracting) of 115 mmHg and diastolic pressure (which is minimum pressure in the arteries, which occurs near the beginning of the cardiac cycle when the ventricles are filled with

blood) of 75 mmHg (commonly written as 115/75 mmHg), cardiovascular disease (CVD) risk doubles for each increment of 20/10 mmHg [47].

A recent classification recommends blood pressure criteria for defining normal blood pressure, prehypertension, hypertension (stages I and II), and isolated systolic hypertension, which is a common occurrence among the elderly. These readings are based on the average of seated blood pressure readings that were properly measured during 2 or more office visits. In individuals older than 50 years, hypertension is considered to be present when a person's blood pressure is consistently at least 140 mmHg systolic or 90 mmHg diastolic. Patients with blood pressures over 130/80 mmHg along with Type 1 or Type 2 diabetes, or kidney disease require further treatment[47].

Classification	Systolic pressure		Diastolic pressure	
	mmHg	kPa	mmHg	kPa
Normal	90–119	12–15.9	60–79	8.0–10.5
Prehypertension	120–139	16.0–18.5	80–89	10.7–11.9
Stage 1	140–159	18.7–21.2	90–99	12.0–13.2
Stage 2	≥160	≥21.3	≥100	≥13.3
Isolated systolic hypertension	≥140	≥18.7	<90	<12.0
Source: American Heart Association (2003).[8]				

Fig. 2 Classification of Systolic and Diastolic Pressure

Hypertension injures blood vessels and thereby causes end-organ damage. The mechanisms are complicated and, although studied for decades in experimental animal models, are only currently being elucidated. It's now in the position of constructing a chain of events from the endothelium to the underlying matrix, to the vascular smooth muscle cells, and beyond to the

adventitia, and surrounding tissues. The endothelial layer acts as a signal transduction interface for hemodynamic forces in the regulation of vascular tone and chronic structural remodelling of arteries. Effects of mechanical forces on signal transduction and gene expression in endothelial cells have been demonstrated. Mechanical stress initiates numerous pathways including ion channels, integrin interaction between cells and matrix, activation of various tyrosine kinases, autocrine production, and release of growth factors. Increased flow through small arteries has been shown to increase connective tissue production and promote medial hypertrophy, probably through proliferation of both endothelial and vascular smooth muscle cells. Increased pressure is also capable of inducing early response genes in the arterial wall. Microvascular endothelium in hypertensive animals has been shown to exhibit increased oxygen radical production attributable to xanthine oxidase. Oxygen radical production by endothelial cells can result in leukocyte-endothelial adhesion responses that involve transcription-independent and -dependent surface expression of different endothelial cell adhesion molecules. Infiltration of the permeabilized endothelium by leukocytes sets the stage for an inflammatory cascade, involving cytokines, chemokines, growth factors, and matrix metalloproteinases. Altered integrin signalling, the production of tenacin, epidermal growth factor signalling, tyrosine phosphorylation, and activation of downstream pathways culminate in vascular smooth muscle cell proliferation. Evidence is accumulating that matrix molecules provide an environment which decreases the rate of programmed cell death.

Mechanical forces alone are capable of initiating complex events resulting in vascular remodelling and subsequent end-organ damage. However, hypertension is not merely a process of mechanical events [37].

3. NAD(P)H OXIDASE POLYMORPHISM AND FOREARM BLOOD FLOW RESPONSES OF WHITE HYPERTENSIVE PATIENTS

Primary (essential) hypertension (PH) is a highly prevalent disease that contributes substantively to the ongoing epidemics of cardiovascular (CV) events in westernized countries[1]. Heritability accounts for over a third of blood pressure (BP) variance, but its mechanisms remain to be fully identified even despite intensive investigation[2]. A blunted endothelium-dependent vasodilatation (EDV),[3] usually termed endothelial dysfunction (ED), is a hallmark of arterial hypertension and atherosclerosis associated conditions[4]. It also predicts cardio-vascular (CV) and cerebro-vascular events independently of the common risk factors,[5-7] thus entailing an intermediate phenotype of PH and, by at large, of CV disease.

Compelling evidence indicates that ED and CV disease are both genetically determined: ED was documented in normotensive offspring of PH parents,[8] and in PH patients carrying an allele (the $^{-786}$C) at a single nucleotide polymorphism (SNP) in the promoter of the endothelial nitric oxide (NO) synthase (eNOS) gene,[9] that implies a blunted gene transcription rate[10]. This allele was also associated with multivessel coronary artery disease (CAD) in Caucasian,[11] and Japanese patients,[10-12] and with increase CV-death free survival. Thus suggesting a link between genetic susceptibility to ED and CV phenotypes.

There are several other plausible candidate genes for ED, among which those of NADH/NADPH oxidases (NAD(P)H)ox, a family of membrane-associated enzymes that catalyze the 1-electron reduction of oxygen using NADH or NADPH as the electron donor. NAD(P)H oxidase are the most important oxidase activity in endothelial (ECs) and vascular smooth muscle cells (VSMCs)[13], and therefore entail a major source of reactive oxygen

species (ROS) in the vasculature where, they can generate the superoxide anion ($O_2^{\circ-}$), in response to haemodynamic forces and hormones[14,15,16].

$O_2^{\circ-}$ scavenges NO, thus leading to ED and peroxynitrite formation; it also activates the NF-$\circledcirc$B-related pathways and is instrumental for inducing CV disease, by triggering adhesins and inflammatory cytokines and by oxidatively modifying LDL[13,17].

In ECs NAD(P)H ox consists of 4 major subunits: 2 cytosolic components, *p47phox* and *p67phox*, and a plasma membrane spanning cytochrome b558 made of a large *gp91phox* and a smaller *p22phox* subunit. The latter entails two histidine residues at position 72 and 94 that are potential heme-binding sites.
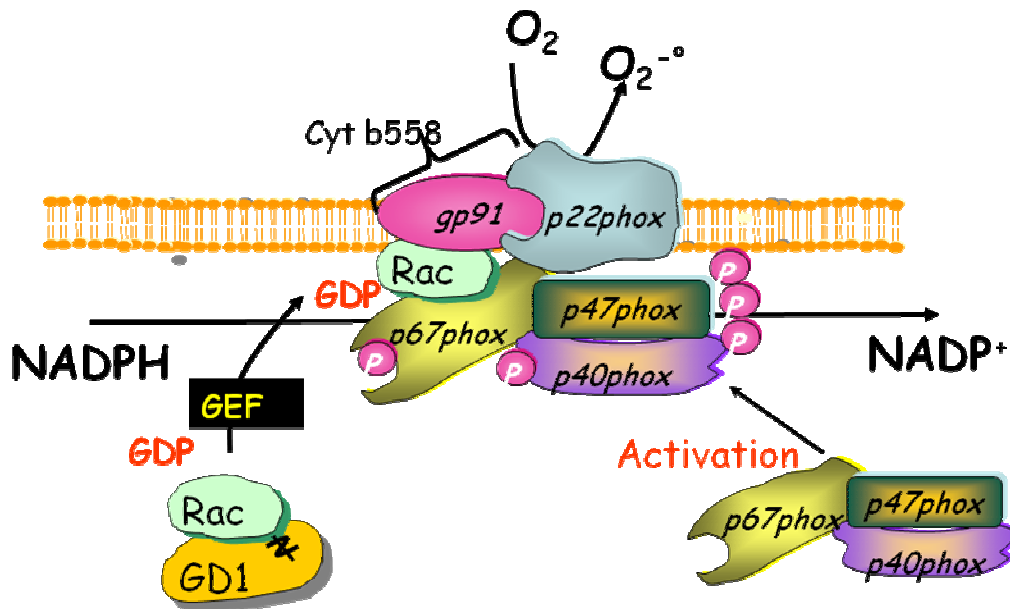

Fig. 3 Structure of NAD(P)H oxidase

Human p22phox is encoded by CYBA gene (according to GenBank Accession Number NM_000101), located on the long arm of chromosome 16 at position (16q24). It spans 7.75 kb and is composed of 6 exons and 5 introns which encode an open reading frame (ORF) of approx. 600 bp.

A significant number of genetic polymorphisms have been reported within the promoter and exonic sequences of the p22*phox* gene, some of which are able to influence gene expression and NADPH oxidase activation, leading to significant functional variation between individuals in oxidative stress. Moreover, some of these polymorphisms have been associated with diverse cardiovascular diseases, such as hypertension, CAD (coronary artery disease), myocardial infarction, cerebrovascular disease, and diabetic and non-diabetic nephropathy [38]. The SNP -930 A/G in the promoter and the $C^{242}T$ located in exon 4 can be functional. The -930 A/G was shown be associated with PH and with hypertension [40]. The ^{242}T allele substitutes histidine-72 to tyrosine residues, thus altering the protein heme-binding properties and, consequently, the NAD(P)H ox activity and regulation. Noteworthy, the expression of the *p22phox* protein, mRNA, and $C^{242}T$ allelic variants ware shown in human blood vessels *ex vivo*, where the ^{242}T allele was associated with reduced vascular NAD(P)H ox activity[18]. These SNPs might therefore affect susceptibility to ED and CV disease by altering $O_2^{\circ-}$ production in human blood vessels[18]. However, even though the ^{242}T allele has been associated with increased EDV responses of epicardial coronary arteries in CAD patients *in vivo*,[19] this finding was inconsistent[20] and could not be replicated in hypercholesterolemic patients[21]. Thus, studies on the functional relevance of these SNPs *in vivo* in humans are conflicting;[22-24] moreover, they did not look upon PH patients. Thus, within a prospective project aimed at discovering the genetic determinants of ED in

uncomplicated PH patients, we sought to investigate if these *p22phox* SNPs influenced EDV trough an interaction with NO bioactivity and vascular $O_2^{\circ-}$ generation.
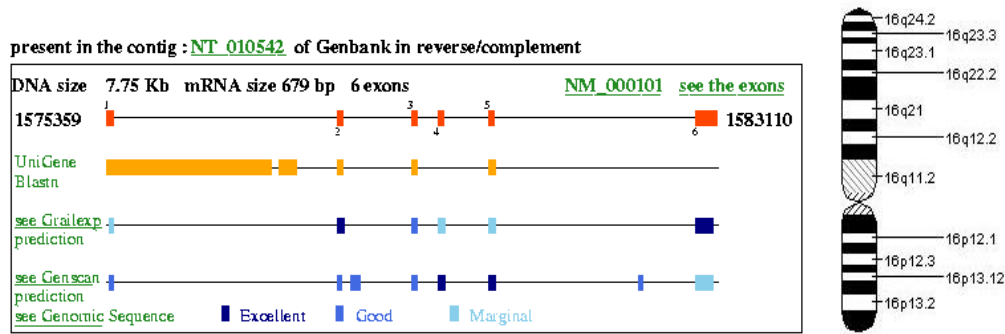

Fig. 4 Structure of CYBA gene and Chromosome 16 on which is placed the gene

4. REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are free radicals that contain the oxygen atom. They are very small molecules that include oxygen ions and peroxides and can be either inorganic or organic. They are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. However, during times of environmental stress (e.g. UV or heat exposure) ROS levels can increase dramatically, which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation.

Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases, catalases, lactoperoxidases, glutathione peroxidases and peroxiredoxins. Small

molecule antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid, and glutathione also play important roles as cellular antioxidants. Similarly, polyphenol antioxidants assist in preventing ROS damage by scavenging free radicals. In contrast, the antioxidant ability of the extracellular space is less, the most important plasma antioxidant in humans is probably uric acid.

Effects of ROS on cell metabolism have been well documented in a variety of species. These include not only roles in apoptosis (programmed cell death), but also positive effects such as the induction of host defence[48] genes and mobilisation of ion transport systems. This is implicating them more frequently with roles in redox signaling or oxidative signaling. In particular, platelets involved in wound repair and blood homeostasis release ROS to recruit additional platelets to sites of injury. These also provide a link to the adaptive immune system via the recruitment of leukocytes.

Reactive oxygen species are implicated in cellular activity to a variety of inflammatory responses including cardiovascular disease. They may also be involved in hearing impairment via cochlear damage induced by elevated sound levels, ototoxicity of drugs such as cisplatin, and in congenital deafness in both animals and humans. Redox signaling is also implicated in mediation of apoptosis or programmed cell death and ischaemic injury. Specific examples include stroke and heart attack[49].

Generally, harmful effects of reactive oxygen species on the cell are most often:

1. damage of DNA

2. oxidations of polydesaturated fatty acids in lipids (lipid peroxidation)

3. oxidations of amino acids in proteins

4. oxidatively inactivate specific enzymes by oxidation of co-factors

Oxidative damage

In aerobic organisms the energy needed to fuel biological functions is produced in the mitochondria via the electron transport chain. In addition to energy, reactive oxygen species (ROS) are produced which have the potential to cause cellular damage. ROS can damage DNA, RNA, and proteins which theoretically contributes to the physiology of ageing.

ROS are produced as a normal product of cellular metabolism. In particular, one major contributor to oxidative damage is hydrogen peroxide (H_2O_2) which is converted from superoxide that leaks from the mitochondria. Within the cell there is catalase and superoxide dismutase that help to minimize the damaging effects of hydrogen peroxide by converting it into oxygen and water, benign molecules, however this conversion is not 100% efficient, and residual peroxides persist in the cell. While ROS are produced as a product of normal cellular functioning, excessive amounts can cause deleterious effects[50]. Memory capabilities decline with age, evident in human degenerative diseases such as Alzheimer's disease which is accompanied by an accumulation of oxidative damage. Current studies demonstrate that the accumulation of ROS can decrease an organism's fitness because oxidative damage is a contributor to senescence. In particular, the accumulation of oxidative damage may lead to cognitive dysfunction as demonstrated in a study where old rats were given mitochondrial metabolites and then given cognitive tests, results showed that the rats performed better after receiving the metabolites, suggesting that the metabolites reduced oxidative damage and improved mitochondrial function[51]. Accumulating oxidative damage can then affect

the efficiency of mitochondria and further increase the rate of ROS production[52]. The accumulation of oxidative damage and its implications for aging depends on the particular tissue type where the damage is occurring. Additional experimental results suggest that oxidative damage is responsible for age related decline in brain functioning. Older gerbils were found to have higher levels of oxidized protein in comparison to younger gerbils. When old and young mice were treated with a spin trapping compound the level of oxidized proteins decreased in older gerbils but did not have an effect on younger gerbils. Additionally, older gerbils performed cognitive tasks better during treatment but ceased functional capacity when treatment was discontinued causing oxidized protein levels to increase. This lead researchers to conclude that oxidation of cellular proteins is potentially important for brain function.

Internal production

Free radicals are also produced inside (and also released towards the cytosol [53,54]) organelles, such as the mitochondrion. Mitochondria convert energy for the cell into a usable form, adenosine triphosphate (ATP). The process in which ATP is produced, called oxidative phosphorylation, involves the transport of protons (hydrogen ions) across the inner mitochondrial membrane by means of the electron transport chain. In the electron transport chain, electrons are passed through a series of proteins via oxidation-reduction reactions, with each acceptor protein along the chain having a greater reduction potential than the last. The last destination for an electron along this chain is an oxygen molecule. Normally the oxygen is reduced to produce water; however, in about 0.1-2% of electrons passing through the chain (this number derives from studies in isolated mitochondria, though the exact rate in live organisms is yet to be fully

agreed upon), oxygen is instead prematurely and incompletely reduced to give the superoxide radical, O_2^-, most well documented for Complex I and Complex III. Superoxide is not particularly reactive by itself, but can inactivate specific enzymes or initiate lipid peroxidation in its H_2O_2 form. The pKa of the protonated superoxide is 4.8, thus at physiological pH the majority will exist as hydrogen peroxide (H_2O_2). If too much damage is caused to its mitochondria, a cell undergoes apoptosis or programmed cell death.

Bcl-2 proteins are layered on the surface of the mitochondria, detect damage, and activate a class of proteins called Bax, which punch holes in the mitochondrial membrane, causing cytochrome C to leak out. This cytochrome C binds to Apaf-1, or apoptotic protease activating factor-1, which is free-floating in the cell's cytoplasm. Using energy from the ATPs in the mitochondrion, the Apaf-1 and cytochrome C bind together to form apoptosomes. The apoptosomes binds to and activates caspase-9, another free-floating protein. The caspase-9 then cleaves the proteins of the mitochondrial membrane, causing it to break down and start a chain reaction of protein denaturation and eventually phagocytosis of the cell.

Cause of aging

According to the Free-radical theory, oxidative damage initiated by reactive oxygen species is a major contributor to the functional decline that is characteristic of aging. While studies in invertebrate models indicate that animals genetically engineered to lack specific antioxidant enzymes (such as SOD) generally show a shortened lifespan (as one would expect from the theory), the converse, increasing the levels of antioxidant enzymes, has yielded inconsistent effects on lifespan (though some well-performed studies in Drosophila do show that lifespan can be increased by the

overexpression of MnSOD or glutathione biosynthesizing enzymes). In mice, the story is somewhat similar. Deleting antioxidant enzymes generally yields shorter lifespan, though overexpression studies have not (with some recent exceptions), consistently extended lifespan[55].

Superoxide dismutase

Superoxide dismutases (SOD) are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. In mammals and most chordates, three forms of superoxide dismutase are present. SOD1 is located in the cytoplasm, SOD2 in the mitochondria and SOD3 is extracellular. The first is a dimer (consists of two units), while the others are tetramers (four subunits). SOD1 and SOD3 contain copper and zinc, while SOD2 has manganese in its reactive centre. The genes are located on chromosomes 21, 6 and 4, respectively (21q22.1, 6q25.3 and 4p15.3-4p15.1).

The SOD-catalysed dismutation of superoxide may be written with the following half-reactions :

- $M^{(n+1)+} - SOD + O_2^- \rightarrow M^{n+} - SOD + O_2$

- $M^{n+} - SOD + O_2^- + 2H^+ \rightarrow M^{(n+1)+} - SOD + H_2O_2$.

where M = Cu (n=1) ; Mn (n=2) ; Fe (n=2) ; Ni (n=2).

In this reaction the oxidation state of the metal cation oscillates between n and n+1.

Catalase, which is concentrated in peroxisomes located next to mitochondria, reacts with the hydrogen peroxide to catalyze the formation of water and oxygen. Glutathione peroxidase reduces hydrogen peroxide by transferring the energy of the reactive peroxides to a very small sulfur

containing protein called glutathione. The selenium contained in these enzymes acts as the reactive center, carrying reactive electrons from the peroxide to the glutathione. Peroxiredoxins also degrade H_2O_2, within the mitochondria, cytosol and nucleus.

- $2\,H_2O_2 \rightarrow 2\,H_2O + O_2$ *(catalase)*

- $2GSH + H_2O_2 \rightarrow GS\text{–}SG + 2H_2O$ *(glutathione peroxidase)*

ROS-directed cancer chemotherapeutics

Recent research demonstrates that redox dysregulation originating from metabolic alterations and dependence on mitogenic and survival signaling through ROS represents a specific vulnerability of malignant cells that can be selectively targeted by pro- and antioxidant redox chemotherapeutics[56].

5. CHEMILUMINESCENCE-BASED ASSAYS

Because of its sensitivity, chemiluminescence is frequently used to detect $O_2^{\circ-}$, in neutrophils and vascular tissue. On exposure to $O_2^{\circ-}$, chemiluminescent probes release a photon, which in turn can be detected by a scintillation counter or a luminometer. Because most of these compounds are cell permeable, the $O_2^{\circ-}$ measured reflects extracellular as

well as intracellular $O_2^{\circ-}$ production. Among these chemiluminescent compounds, bis-*N*-methylacridinium nitrate (lucigenin) remains the most widely used. Other compounds that may be used include 5-amino-2,3-dihydroxy-1,4-phthalayineidone (luminol) and cypridina luciferin analogues, such as 2-methyl-6-phenyl-3,7-dihydroimidazo (1,2-α)-pyrazin3-

one (CLA), 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo(1,2-α)pyrazin-3-one (MCLA), and 2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl) 8-benzyl-3,7-dihydroimidazol[1,2-α]pyrazin-3-one (coelenterazine).

Luminol

Luminol-derived chemiluminescence may be evoked by a variety of reactive oxygen species, including $O_2^{\circ-}$, hydroxyl radical, hydrogen peroxide, and peroxynitrite. Thus, the selective use of various scavengers, such as ebselen, uric acid, catalase, and SOD, is necessary to determine which of these species is responsible for the signal produced by luminol. For example, a recent study has used the scavengers uric acid to show that peroxynitrite formation is markedly elevated in the aortas of apoE-deficient mice.

Fluorescence-Based Assays

During the past several years, a number of groups have used fluorescent probes to detect reactive oxygen species in cultured cells and in sections of vascular tissues. In cultured cells, confocal microscopy has been used to directly image the fluorescence produced by these agents. These immunofluorescence approaches are semiquantitative but can provide important information about the topographical location of reactive oxygen species in the vessel wall. Cells can also be analyzed with the use of a fluorescent plate reader or by fluorescence-activated cell sorter counting to provide quantitative information. Even when these more quantitative approaches are used, these fluorescent probes are subject to quenching and may not increase linearly as the levels of reactive oxygen species increase.

6. ENDOTHELIAL CELLS AND ENDOTHELIAL DYSFUNCTION

The endothelium is the thin layer of cells that line the interior surface of blood vessels[61], forming an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells (ECs) line the entire circulatory system, from the heart to the smallest capillary. These cells reduce turbulence of the flow of blood allowing the fluid to be pumped farther. Endothelial tissue is a specialized type of epithelium tissue (one of the four types of biological tissue in animals). More specifically, it is simple squamous epithelium.

Endothelial cells are involved in many aspects of vascular biology, including:

- Vasoconstriction and vasodilation, and hence the control of blood pressure
- Blood clotting (thrombosis & fibrinolysis)
- Atherosclerosis
- Formation of new blood vessels (angiogenesis)
- Inflammation
- Barrier function - the endothelium acts as a selective barrier between the vessel lumen and surrounding tissue, controlling the passage of materials and the transit of white blood cells into and out of the bloodstream. Excessive or prolonged increases in permeability of the endothelial monolayer, as in cases of chronic inflammation, may lead to tissue edema/swelling.

In some organs, there are highly differentiated endothelial cells to perform specialized "filtering" functions. Examples of such unique endothelial

structures include the renal glomerulus and the blood-brain barrier. Endothelial dysfunction (ED) is a systemic pathological state of the endothelium (the inner lining of our blood vessels) and can be broadly defined as an imbalance between vasodilating and vasoconstricting substances produced by (or acting on) the endothelium[61]. Normal functions of endothelial cells include mediation of coagulation, platelet adhesion, immune function, control of volume and electrolyte content of the intravascular and extravascular spaces. Endothelial dysfunction can result from and/or contribute to several disease processes, as occurs in septic shock, hypertension, hypercholesterolaemia, diabetes as well from environmental factors, such as from smoking tobacco products and exposure to air pollution. Endothelial dysfunction is thought to be a key event in the development of atherosclerosis and predates clinically obvious vascular pathology by many years. This is because endothelial dysfunction is associated with reduced anticoagulant properties as well as increased adhesion molecule expression, chemokine and other cytokine release, and reactive oxygen species production from the endothelium, all of which play important roles in the development of atherosclerosis. In fact, endothelial dysfunction has been shown to be of prognostic significance in predicting vascular events including stroke and heart attacks. Because of this, endothelial function testing may have great potential prognostic value for the detection of cardiovascular disease, but currently the available tests are too difficult, expensive, and/or variable for routine clinical use. A key and quantifiable feature of endothelial dysfunction is the inability of arteries and arterioles to dilate fully in response to an appropriate stimulus that stimulates release of vasodilators from the endothelium like nitric oxide (NO). Endothelial dysfunction is commonly associated with decreased NO

bioavailability, which is due to impaired NO production by the endothelium and/or increased inactivation of NO by reactive oxygen species. This can be tested by a variety of methods including ionophoresis of acetylcholine, direct administration of various vasoactive agents to segments of blood vessels, localized heating of the skin and temporary arterial occlusion by inflating a blood pressure cuff to high pressures. Testing can also take place in the coronary arteries themselves but this is invasive and not normally conducted unless there is a clinical reason for intracoronary catheterisation. Of all the current tests employed in the research setting, flow-mediated dilation is the most widely used non-invasive test for assessing endothelial function. This technique measures endothelial function by inducing reactive hyperemic via temporary arterial occlusion and measuring the resultant relative increase in blood vessel diameter via ultrasound. As people with endothelial dysfunction have low NO bioavailability, their blood vessels have a decreased capacity to dilate in response to certain stimuli, compared to those with normal endothelial function. Because NO has anti-inflammatory and anti-proliferative effects and therefore helps inhibit atherosclerosis, it is easy to see how endothelial dysfunction may contribute to future adverse cardiovascular events. Unfortunately the variability in such tests (e.g. due to time of day, food, menstrual cycle, temperature, etc.) means that no technique has yet been identified that would allow endothelial testing to attain routine clinical significance, although there are some tests under clinical evaluation such as measuring of arterial stiffness. As more research is conducted to improve testing methodologies for endothelial dysfunction, however, several non-invasive tests have been created by various medical-research companies[62]. Endothelial function can be improved significantly by exercise and

improved diet. A study published in 2005 has determined that a positive relationship exists between the consumption of trans fat (commonly found in hydrogenated products such as margarine) and the development of endothelial dysfunction[63]. Other factors have been identified as improving endothelial function and include cessation of smoking, loss of weight and treatment of hypertension and hypercholesterolemia amongst other things. Some studies have found antioxidant and arginine supplementation to restore impaired endothelial dysfunction. Endothelial dysfunction has been observed in a 2001 study of women where it was found that this disorder is present in approximately half of women with chest pain, in the absence of overt blockages in large coronary arteries. This endothelial dysfunction cannot be predicted by typical risk factors for atherosclerosis (e.g., obesity, cholesterol, smoking) and hormones[64].

7. SINGLE NUCLEOTIDE POLYMORPHISM (SNP)

A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide — A, T, C, or G — in the genome differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide. In this case we say that there are two *alleles* : C and T. Almost all common SNPs have only two alleles. Within a population, SNPs can be assigned a minor allele frequency. This is simply the lesser of the two allele frequencies for single-nucleotide polymorphisms. There are variations between human populations, so a SNP allele that is common in one geographical or ethnic group may be much rarer in another. Single

nucleotides may be changed (substitution), removed (deletions) or added (insertion) to a polynucleotide sequence. Ins/del SNP may shift translational frame[57,58,59]. Single nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed *synonymous* (sometimes called a silent mutation) if a different polypeptide sequence is produced they are *nonsynonymous*. A nonsynonymous change may either be missense or nonsense, where a missense change results in a different amino acid, while a nonsense change results in a premature stop codon. SNPs that are not in protein-coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA. Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens, chemicals, drugs, vaccines, and other agents. SNPs are also thought to be key enablers in realizing the concept of personalized medicine[60]. However, their greatest importance in biomedical research is for comparing regions of the genome between cohorts (such as with matched cohorts with and without a disease).

AIMS OF THE STUDY

The main goal of our study was to investigate the quantitative expression of $O_2^\circ{-}$ in patients with different SNPs of p22phox to investigate the potential role of this ROS in the cardiovascular disease.

Specific objectives were to investigate:

1. if the effect of two single nucleotide polymorphisms (SNPs) in the promoter and in exon4 of CYBA gene corresponds to one different production of $O_2^\circ{-}$, involved in oxidative stress;

2. if the effect of alleles at two identified single nucleotide polymorphisms (SNPs) in the promoter (-930 A / G) and in exon 4 (C242T) of CYBA gene (NC_000016.8), which encode for p22phox subunit of NAD(P)H oxidase, affect endothelium-dependent and endothelium-independent vasodilatation in vivo in healthy normotensive human subjects and in patients with essential hypertension;

3. if the mentioned polymorphisms modify half life of NAD(P)H oxidase enzyme.

MATERIALS AND METHODS

The major difficulty of this study was to develop the design of probes and primers for PCR. The area where you find the mutation is, in fact, a rich zone in CG which tends to create mismatch. Probes created by Zalba et al. and used in our laboratory didn't give amplification.

Study design and Enrolements

Two different studies were conducted: one in our laboratory and the other in collaboration with Departments of Internal Medicine, University of Pisa.

Patients enrolled (n=15) in the first study, were patients undergoing revascularization after carotid occlusion by vascular surgery. The criteria for exclusion from the study were the presence of viral infectious diseases. This was the real problem of the study as many of the operated patients showed suspected or confirmed disease.

The endothelial cells were extracted from jugular veins of this patients and then were assessed for production of superoxide. The blood was collected in buffy coat and was used to determine two single nucleotide polymorphisms (SNPs) in the promoter and in exon4 of CYBA gene.

In the second study, 195 primary hypertensive patients and 71 healthy normotensive volunteers participated in the study, which was approved by the Medical Ethics Committee of the Universities of Pisa and Padova. Subjects with hypercholesterolemia (total cholesterol $\geq$ 5.2 mmol/L), diabetes mellitus, cardiac and/or cerebral ischemic vascular disease, impaired renal function, and other major pathologies were excluded from

the study. In accordance with institutional guidelines, all patients were aware of the investigational nature of the study and gave written consent.

Subjects were defined as normotensive according to the absence of family history of essential hypertension and BP values of ≤ 140/90 mm Hg. Normotensive subjects were recruited among the individuals afferent to our department provided they had demographic characteristics comparable to those of hypertensive patients. Primary hypertensive patients were recruited from among the newly diagnosed patients in the outpatient clinic of the Department of Internal Medicine of the University of Pisa if they reported a positive family history of essential hypertension, whenever supine arterial BP (after 10 minutes of rest) measured with mercury sphygmomanometry (with phase V Korotkoff), 3 times at 1-week intervals for 1 month, was consistently found to be ≥ 140/90 mm Hg. The vast majority (> 80%) of PH patients had never been treated, the rest reported a history of discontinued (for at least 6 months) or ineffective pharmacological treatment[25]. Secondary forms of hypertension were excluded by routine diagnostic procedures;[26] pharmacological treatment was withdrawn at least two weeks before performing the study.

Patients enrolled in the study for superoxide dosage were undergoing patients (n=15) to revascularization after carotid occlusion, and then came from vascular surgery. The criteria for exclusion from the study were those concerning viral infectious diseases. This was the real problem of this study because many operated patients, showed suspected or confirmed viral disease.

Experimental procedure

A cannula was inserted into the brachial artery for drug infusion at systemically ineffective rates, while intra-arterial BP and heart rate were monitored. FBF was measured in both forearms by strain-gauge venous plethysmography[9,25]. Circulation to the hand was excluded one min before FBF measurement by inflating a pediatric cuff around the wrist at supra-systolic BP.

EDV was estimated with a dose-response curve to intra-arterial acetylcholine (ACH) (cumulative increase of the infusion rates: 0.15, 0.45, 1.5, 4.5, 15 μg/100 ml forearm tissue/min, for 5 min at each dose) while endothelium-independent vasodilatation (EIV) was assessed with intra-arterial infusion of sodium nitroprusside (SP) (cumulative increase by 1, 2 and 4μg/100 ml forearm tissue/min, for 5 min at each dose). These rates were selected to induce vasodilatation comparable to that obtained with ACH. The ACH or SP infusion sequence was randomized; 30-min washout was allowed between each dose-response curve.

To further investigate the impact of the *p22phox* genotype on ACH-induced NO-mediated EDV in 163 subjects (111 HT and 52NT) we co-administered the NOS inhibitor N(G)-monomethyl-L- arginine (L-NMMA, 100 mg/100 mL forearm tissue/min); in 91 subjects (56 HT and 35 NT) we verified whether this SNP affected the inactivation of NO by ROS with an intrabrachial infusion of vitamin C (2.4 mg/100 mL forearm tissue/min).[27]

Extraction of DNA and genotyping

The blood was collected in EDTA and stored at -20°C until DNA was extracted according to standard procedures using a commercially available

kit (DNA Blood Extraction Fast KIT,™ AB Analitica Srl, Italy)[35;36] and quantified by spectrophotometer and genotyping by allele-specific Taqman PCR.

Genotyping

FRET methodology

For genotyping of the SNP C242T we designed four oligonucleotides: two serving as amplification primers and two as fluorescence resonance energy transfer (FRET) probes. The acceptor and the donor probe were labelled at the 5' end with a red fluorophore (LCRed640) and at the 3'-end with fluorescein, respectively.

Forward   5'-GCAACCCTCAACAGCTCCT-3'

Reverse   5'-CAGCAGAGTCCCAATGGTG-3'

Probe LCRed640   5'-GACAGAAGTACATGACCGCCGT-3'

Probe FL   5'-GAAGCTGTTCGGGCCCTTTACCA-3'

Probe/DNA hybrids that contain a mismatch melt at lower temperatures than perfectly matched hybrids, thus resulting in different Tm during melting curve analysis. For this analysis, at the end of PCR, temperature was raised to 94°C, lowered to 45°C and then slowly raised to 78°C to allow monitoring of the decline of fluorescence generated by melting of the hybrids, as a function of temperature. Melting curves were automatically converted to fluorescence peaks. Hence, distinction of wild type, mutant, homozygous and of heterozygous genotype can be easily accomplished by differences in their respective Tm. This methodology was found to be 100% accurate vs sequencing for genotyping at this polymorphism. The PCR reaction mixture consisted of Lightcycler DNA Master Hybridization Probes 10x (Roche Diagnostics, Milan, Italy), to which primers (0.5 µM), and

probes (0.15 µM) were added. The cycling program entailed a denaturation step (95°C for 2 min) followed by 50 cycles as follows: 94°C-5 s, 58°C-15 s, 72°C 12 s. The sequence of amplification primers and FRET probes are available from the corresponding author upon request.

TaqMan Methodology

This PCR method is based on the accumulation of fluorescence during the nucleotidic degradation of an internally quenched allele-specific probe. However, probes that differ from the template sequence by as little as a single nucleotide exhibit a lower melting point temperature and remain intact during PCR. They will be displaced from the template strand but not degraded by the esonuclease activity of Taq polymerase. The correct extension temperature is crucial for the performance of the assay. Allelic discrimination is achieved by the use of two oligonucleotide probes, each complementary to one of the two alleles and labeled with a different fluorescent reporter dye. The design of suitable probes is dependent on the sequence of the two alleles.

We designed specific primers and probes corresponding to position of that sequence for the allelic discrimination assay to investigate two different single nucleotide polymorphisms (SNPs) in the promoter (-930 A / G) of CYBA gene.

The TaqMan probe for detection of the SNP -930 A/G allele 1 (5′- AGCATT **G**CTGCCTCCG-3′) was 5′-labeled with the reporter dye 6 - carboxyfluorescein (FAM), the TaqMan probe for the allele 2 (5′-CCA GCATT**A**CTGCCT CC-3′) was 5′-labeled with CY5. The underlined nucleotides indicate the position of the base exchange of the TaqMan polymorphism. The primers 5′-GAATGGTGGCAGGAGTG-3′ (forward)

and 5'-GGTTTACGAAACAGAAAAACGG-3' (reverse) were used for amplification. The primers and TaqMan probes were synthesized by TIB Molbiol. The PCR reaction was performed in a volume of 20 μl containing 18 μl of PCR reaction mixture that consisted of Lightcycler Fast Start TaqMan Probes Master Hybridization 10x (Roche Diagnostics, Milan, Italy), to which primers (0.05 μM), and probes (0.05 μM) were added.

The cycling program entailed a denaturation step (95°C for 5 min) followed by 50 cycles of amplification as follows: 95°C – 10sec, 60°C – 7sec, 72°C 6sec and a cooling step (40°C for 1 min). Analysis of the data were performed with the LightCycler 480 (Roche Diagnostics, Milan, Italy).

	SNP - 930 A/G	Accession number	Amplicon
Primers	Sequence	NM_000101	100 bp
Forward	5'-GAATGGTGGCAGGAGTG-3'		
Reverse	5'-GGTTTACGAAACAGAAAAACGG-3'		
Probe FAM	5'- AGCATT **G**CTGCCTCCG-3'		
Probe CY-5	5'-CCAGCATT**A**CTGCCTCC-3'		
	SNP C242T		348 bp
Forward	5'-GCAACCCTCAACAGCTCCT-3'		
Reverse	5'-CAGCAGAGTCCCAATGGTG-3'		
Probe LCRed640	5'-GACAGAAG**T**ACATGACCGCCGT-3'		
Probe FL	5'-GAAGCTGTTCGGGCCCTTTACCA-3'		

Table 1. RT-PCR primers and PCR products

Sequencing

To determine and verify the exact DNA polymorphism, we first amplified DNA samples by PCR with the primers and conditions as described above (genotyping). The PCR products (40 mL) were subsequently purified using the QIAquick PCR purification method (QIAGEN), and the DNA concentration was determined. DNA (400 ng) was sequenced in each direction by dye-terminator chemistry using a DNA sequencer (Applied Biosystems).
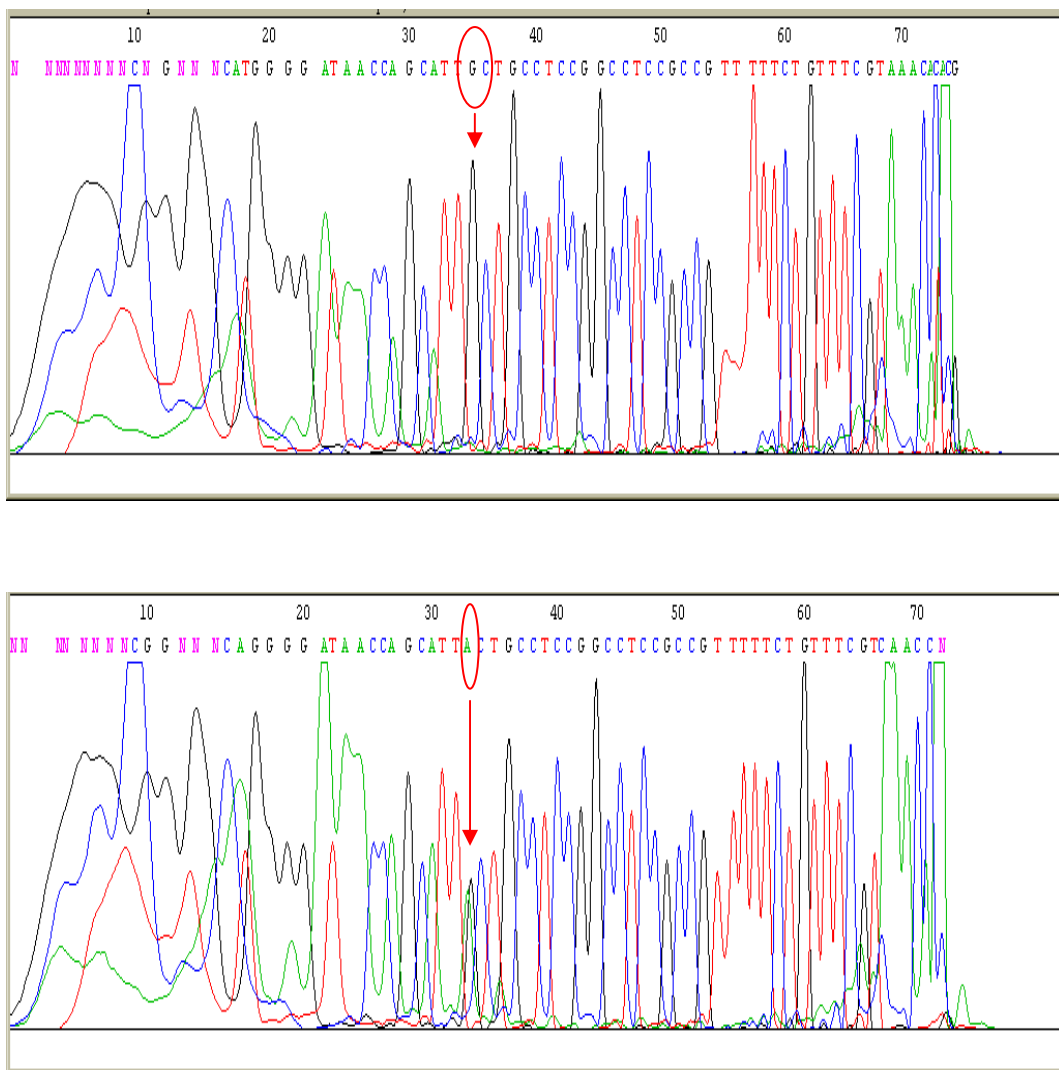

Fig.5 Sequencing of DNA fragment on which is located the SNP -930A/G. The first electropherograms showed a genotype GG and the second showed genotype AG

Cells extraction and immunoseparation

Cell pool was detached from veins whit collagenase (20 min a 37°C). Endothelial cells isolation from pool were done whit Dynabeads® CD31 Endothelial Cell (Invitrogen).

Dynabeads CD31 are uniform, superparamagnetic polystyrene beads (4.5 µm diameter) coated with a mouse IgG1 monoclonal antibody specific for the CD31 cell surface antigen PECAM-1 (platelet endothelial cell adhesion molecule-1). The primary CD31 antibody is attached to the Dynabeads via a secondary antibody to ensure optimal orientation of the primary antibody. The secondary antibody on the Dynabeads is a human IgG4 anti-mouse IgG. The source of the human monoclonal antibody is free of Human Immunodeficiency Virus (HIV), Hepatitis-B Virus (HBV) and Hepatitis-C Virus (HCV).

Dynabeads were mixed with the cell sample in a tube. The Dynabeads will bind to the target cells during a short incubation, and then the beadbound cells were separated by a magnet. The positive isolation was to discard the supernatant and used the bead-bound cells for downstream applications. Positively isolated endothelial cells were pure, viable and unstimulated and were ideal for culture with the Dynabeads still attached. Fibroblast, pericyte or smooth muscle cell contamination was avoided. The endothelial cells will grow well and adhere normally and after approximately three passages, all the beads were diluted out.

At the cell suspension was added the appropriate volume of Dynabeads, incubated for 1 hours at 4°C with gentle tilting and rotation.

Paste the hour placed the tube in a magnet for 3 min, discarded the supernatant and washed the bead-bound cells 3 times by resuspending in Buffer 1 to the original sample volume, and separate using a magnet. The

bead-bound endothelial cells were now ready for plating in a plate 24 well whit growth medium. The Growth Medium contains: FCS-10, ECGS/H-2, Hegf-0.05, HC-500 (PromoCell), antibiotic (Sigma).

Fig. 6 Endothelial Cells Isolated with beads antiCD31
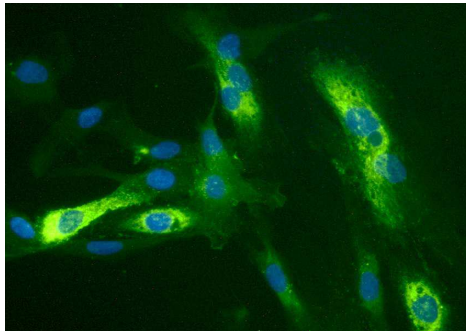
Immunostaining for von Willebrand factor

To assess whether the isolated cells were endothelial cells, was practiced immunostaining for von Willebrand factor.

The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI). No fluorescence was observed in negative control.

For immunostaining, cells were fixed in acetone (–20°C) for 3 min, blocked with PBS, 0.3% Triton X-100, and 1% BSA for 1 h, and incubated rabbit anti-human von Willebrand factor (vWF) antibody in PBS, 0.3% Triton X-100, and 1% BSA at 4°C overnight. After being washed 3 times with PBS, cells
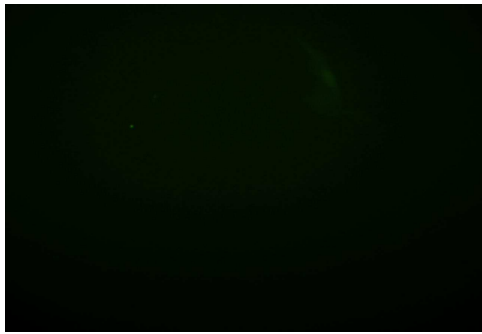
were incubated with anti-rabbit FITC secondary antibody for 40 min at room temperature, followed by three washes with PBS (Fig.7).

A
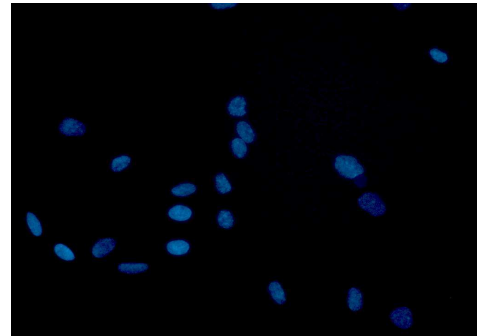
B

C

D

Fig.7 A: Immunostaining for von Willebrand factor (green) and 4′, 6-diamidino-2-phenylindole (DAPI) (blu) expressed on endothelial cells. B: Immunostaining for nucleus of endothelial cells with DAPI. C: negative control. D: ImmunoIstochemestry with DAB

Measurement of NADPH Oxidase Activity

After 2-3 week, the endothelial cells were ready for superoxide dosage by a luminometer designed as a universal microplate. Due to its sophisticated measurement technology and user-friendliness it is particularly suited for all bio- and chemiluminescence measurements.

The instrument works with an extremely low-noise photomultiplier. The highest sensitivity possible is obtained due to the use of single photon counting technology. The measurement accuracy is further enhanced by using special crosstalk reduction methods. The kinetics of fast luminescence reactions can be traced accurately by using an ultra fast amplifier.

Superoxide produced by the endothelial cells was determined using an luminol-enhanced chemiluminescence assay. Oxidant production was inhibited by superoxide dismutase.

Preparation of Reagents for the Superoxide Anion Assay (Calbiochem® Superoxide Anion Assay Kit)

5 µl of 4 mM luminol solution were diluted with 89 µl of Superoxide Anion assay medium. Were added 5 µl of 5 mM enhancer solution to the mixture of luminol and Superoxide Anion assay medium. 1 mg/ml solution of Phorbol-12-myristate-13-acetate (PMA) in DMSO was prepared and vortexed vigorously until the PMA dissolves. To prevent PMA hydrolysis in water, this solution was diluted to a concentration of 20 µg/ml PMA with chilled 150 mM NaCl. The diluted PMA solution was vortexed for 15 s. This solution is known as the SOA Assay Medium Reagent Mixture.

Endothelial cells (1 x10^5) were collected in a conical tube by centrifuging cell suspension at 3000 x g for 5 min, were resuspended in 1 ml of fresh supplemented growth medium to increase the reactivity of the cells. The cells were incubated for 30 min at 37°C, spined the tube in a microcentrifuge at 1600 rpm for 2 min, removed and discarded the supernatant, resuspended the cells in 100 µl of Superoxide Anion assay medium. 2 µl of the diluted PMA solution was added to the mixture of

luminol, enhancer, and Superoxide Anion assay medium and incubated for 20 min at room temperature. After 20 min, chemiluminescence (CL) was measured at room temperature in a Centro LB 960 luminometer. The CL was continually recorded at 30 min intervals for 360 min.

Statistical analysis

A regression analysis was used to identify significant predictors of FBF responses to ACH or SP, which were then entered as covariates in a model for repeated measures ANOVA for the purpose of group's comparison. Interaction terms between $C^{242}T$ SNP, age, gender, and hypertension were also used in this model. To detect significant effects, we decided *a priori* to use of a multivariate test (Roy's largest root), because it does not require the sphericity assumption. Unpaired t-test was used for comparison of the demographic and haemodynamic variables between PH and NT groups; one-way ANOVA followed by Bonferroni post-hoc test was used to locate significant differences across genotypes. A generalized linear model was used to compare covariate-adjusted maximal FBF response to ACH, SP, L-NMMA, and vitamin C between NT and PH groups and across *p22phox* genotypes.

Linkage disequilibrium (LD) between the two SNPs was estimated using both D′ and r^2(Haploview, http://www.hapmap.org). The two SNPs, -930 A/G and C242T were not in LD.

For the analysis of the genotype effect on ROS generation *in vitro* we used the non-parametric Friedmann and Mann-Whitney tests.

Statistical significance was set a p < 0.05. Analyses were carried out with the SPSS for Windows™ statistical package (vers.18.0, SPSS Inc, Bologna, Italy).

RESULTS

Demographic characteristics and p22phox genotypes

The demographic and clinical characteristics of the patients are shown in Table 2.

The patient's group were composed of 4 subjects with genotype GG/CC, 1 subject with genotype GG/TT, 2 subjects with genotype AG/TT, 1 subject with genotype AG/CC, 2 subjects with genotype AG/CT, 1 subject with genotype AG/CC, 3 subjects with genotype AA/CT, 1 subject with genotype AA/TT.

The -930 A/G and $C^{242}T$ *p22phox* genotypes distribution was consistent with the Hardy-Weinberg equilibrium.

Age (year)	65 ±11
Gender (♂:♀)	9:6
Systolic blood pressure (mmHg)	170±16
Diastolic blood pressure (mmHg)	80±11
Serum K^+ concentration(mmol/L) [3.6-4.5]	3.3±0.6
BMI (Kg/m^2)	25.2 ± 3.5
Polymorphism -930A/G	5GG; 6AG; 4AA
Polymorphism C242T	6CC; 5CT; 4TT

Table 2. Anthropometric, biochemical features and genotype of the patients.

Superoxide Production

Data obtained from the assay, showed that only 4 patients (number 2, 6, 14 and 15) have a production of superoxide about forty times higher than other at 300 min after the first measurement, as shown in Fig. 8.

These patients are exactly those with genotype GG/CC for SNPs -930A/G and C242T respectively.
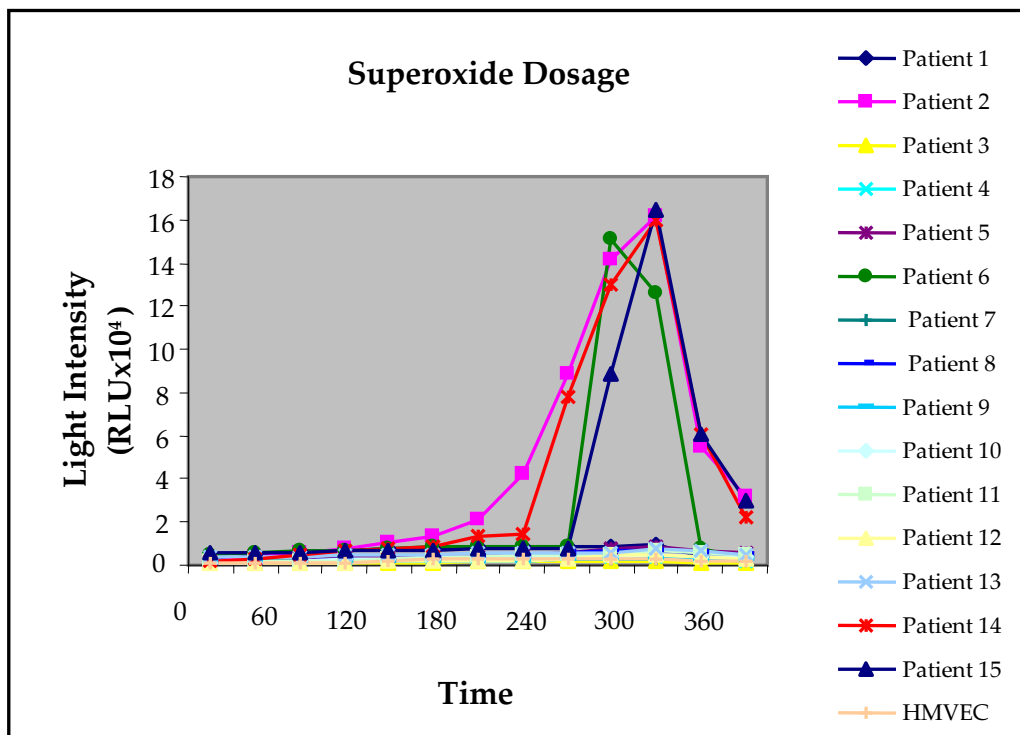

Fig. 8 Measurement of chemiluminescence derived by Superoxide production in patients and in Human Dermal Microvascular Endothelial Cells (HMVEC).
The light was detected in the assay for superoxide anion activity during a 360 minutes period and was measured in relative light units (RLU). Superoxide levels increased after 300 min from the first measurement only in patients with genotype GG/CC

Relation between the p22phox polymorphism and Endothelial Cells NADPH Oxidase Activity

NADPH oxidase activity was similar in all subjects except in the patients with GG/CC genotype (Fig. 9).
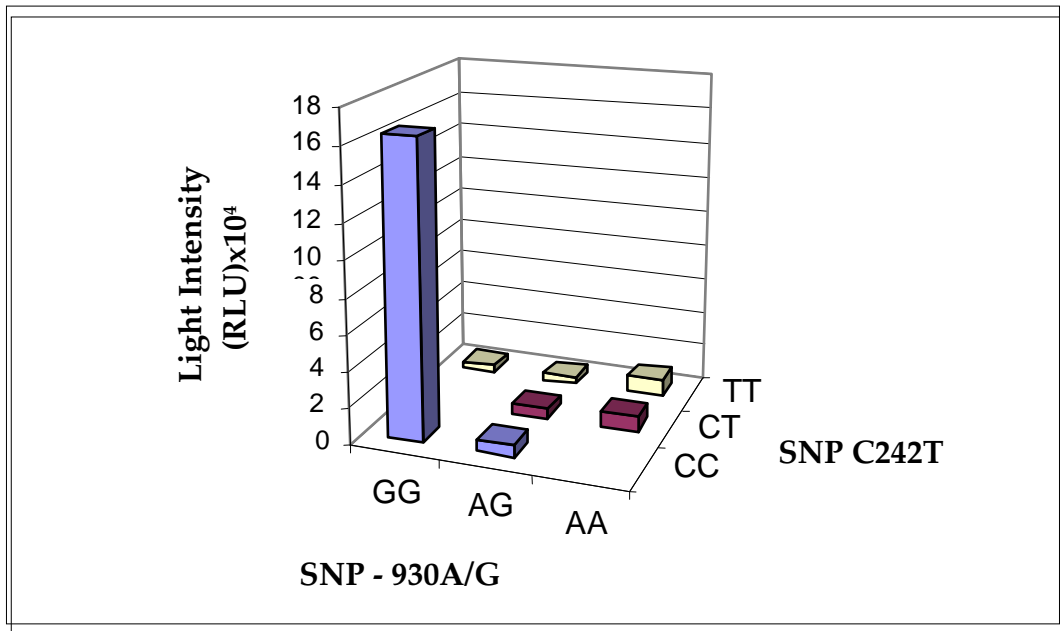
Fig.9 Detection of increased endothelial Superoxide in genotype GG/CC. Luminol chemiluminescence was measured in RLU

The experiment was repeated after about a month on the same endothelial cells, but the results were not satisfactory and this was probably due to the undifferention cells.

Endothelium-dependent vasodilatation (EDV)

Baseline FBF on the occasion of both the ACH and the SP study were similar in PH patients and NT subjects and across the -930 A/G and $C^{242}T$ genotypes. ACH induced a significant increase of FBF in both PH patients and NT subjects (Fig. 10); at the maximum ACH dosage, this increase was markedly blunted ($p<0.001$) in PH compared to NT subjects. The maximal FBF response to ACH was significant affected by hypertension, triglycerides, gender, and smoking, which overall accounted for 17% of its variance (adjusted $R^2=0.17$, $p=0.001$). Therefore, these variables were entered as covariates in the repeated measures ANOVA model.

Besides the significant effect of hypertension ($p<0.001$) on EDV, there was also an effect of the $C^{242}T$ p22phox SNP per se ($p=0.014$) and through an interaction with hypertension ($p<0.001$), hypertension and gender ($p<0.001$), smoking ($p<0.001$), and with gender and smoking ($p<0.001$), When PH patients and NT subjects were analyzed separately for EDV, a significant effect of the p22phox SNP per se was seen. Of much interest the CT heterozygous showed a significantly higher EDV, compared to the other genotypes, in the NT and also in the PH groups.
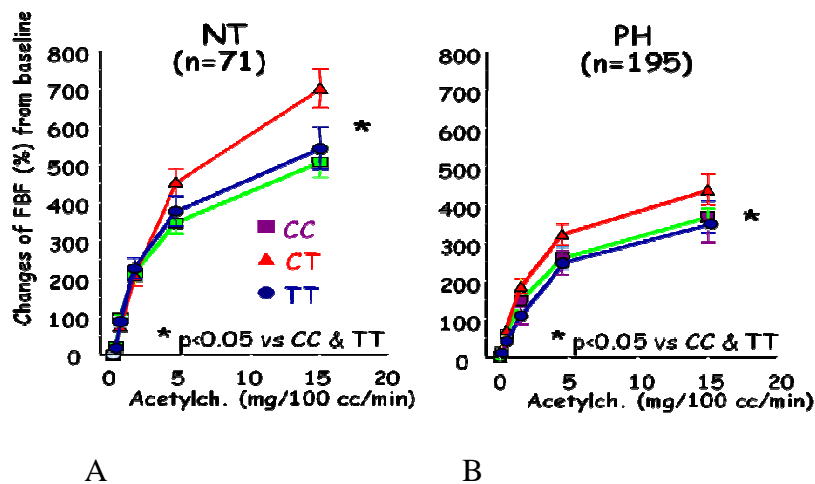
Fig.10 The graphs show the FBF responses (% increase from baseline) to an intra-brachial artery infusion of the E-dependent vasodilator acetylcholine in the normotensive (NT) subjects (A panel), and in the primary hypertensive (PH) patients (B panel) divided according to the p22phox $C^{242}T$ NADPH ox SNP. The increase of FBF was significantly (p<0.001) blunted in the PH compared to the NT subgroup. A significant effect of to the p22phox $C^{242}T$ NADPHox SNP was seen in the all study population as well as in the HT and PH subgroups: in both the CT heterozygous (triangles) showed a significantly higher (** p<0.01; * p<0.05) FBF than the CC (squares) and TT (circles) homozygous

Endothelium-independent vasodilatation (EIV)

SP significantly increased FBF in both PH patients and NT subjects, without differences between groups at any dosages. Regression analysis identified BMI, smoking, gender, LVMI, HDL-cholesterol and triglycerides as significant predictors of the FBF response to SP. Accordingly, these covariates, which explained about 20% of FBF response variance (adjusted R^2=0.20, p=0.002), were used in the repeated measures ANOVA model. No significant effect of the p22phox SNP and no significant interactions on EIV were detected (not shown) in the overall study population, or in any subgroup.

Effect of NO inhibition and of $O_2^{\circ-}$ scavenging

The administration of L-NMMA markedly blunted the FBF response to ACH in the NT subjects to a significantly (p<0.001) smaller extent than in the PH patients (by 53.8±4.2%, m±sem vs 2.5±1.6%, respectively). The administration of vitamin C markedly improved the FBF response to ACH in the PH patients (+63.5±6.0%), but not in the NT subjects (+2.7±4.6, p<0.001 between groups). Therefore, a highly significant inverse correlation (Spearman r = 0.765, p<0.001) between the changes of FBF induced by NO inhibition and by $O_2^{\circ-}$ scavenging was observed (Fig.11).

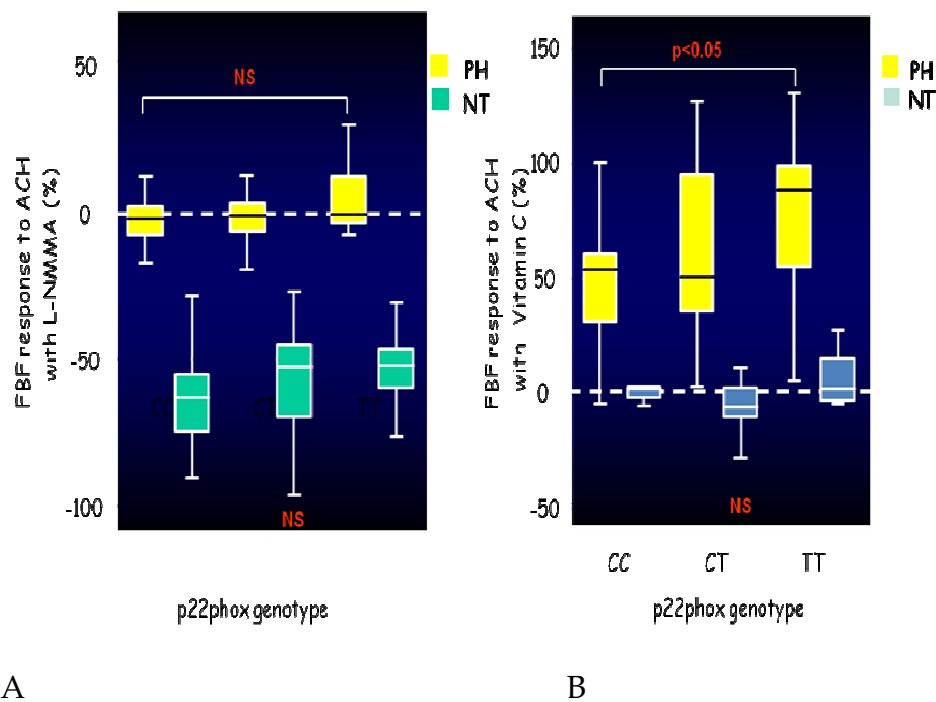A   B

Fig.11 The graph A shows the median value, interquartile range (box), and range of FBF responses to ACH induced by L-NMMA in the PH and NT groups classified by p22phox genotype. A lower decrease of EDV with NO inhibition was seen in the TT compared to the CC individuals.

The graph B shows the median, interquartile range and extreme values of the change in FBF responses to ACH induced by Vitamin C in the PH and NT groups classified by p22phox genotype. No significant differences across genotypes were observed

Effect of the p22phox -930A/G and C242T genotype on NO inhibition and of $O_2^{\circ-}$ scavenging

When PH patients and NT subjects were divided by $C^{242}T$ and -930 A/G p22phox SNP a significantly effect of the SNP on responses of FBF to NO inhibition was seen ($p<0.05$). Comparison across genotypes showed that the TT homozygous had a significantly smaller blunting of EDV than the CC homozygous, albeit they did no differ significantly from the CT heterozygous. However, this difference was more marked in the NT than in the PH group.

At variance, only a trend ($p=0.07$) toward a greater EDV response to vitamin C in the TT homozygous compared to either CC ($p=0.075$) or CT ($p=0.076$) was found both in the PH and in the NT group.

Of interest, the correlation between changes of FBF in response to ACH during NO inhibition and after scavenging of $O_2^{\circ-}$ was consistent across all p22phox genotypes.

DISCUSSION

In the study conducted in our laboratory and in collaboration with the Departments of 2 Internal Medicine, University of Pisa, we have sought to determine if the -930A/G and the $C^{242}T$ SNP of the *p22phox* gene that encodes a subunit of the major $O_2^{\circ-}$ forming enzyme in ECs, NAD(P)H ox, affected the EDV and EIV in the human forearm vascular bed, as assessed by established pharmacological techniques. These variant SNPs ware chosen, among others, because they ware shown to affect vascular NAD(P)H ox activity in human vessels *in vitro*,[18] thus suggesting a functional relevance *in vivo*. Investigation of a fairly large population of uncomplicated mild-to-moderate PH, most of whom had never been previously treated, and a control group of healthy normotensive subjects provided a reasonable statistical power to this study.

Effect of the -930A/G and $T^{242}C$ p22phox polymorphism on EDV (Endothelium-Dependent vasodilatation) and EIV (Endothelium-Independent vasodilatation)

The present findings confirmed previous results that documented a blunted FBF EDV responses and similar EIV responses in PH patients compared to NT subjects[28]. More importantly, they provide novel information on the import of the -930A/G and $C^{242}T$ *p22phox* SNP on EDV, an area of research where conflicting data were available[19,21,23,29,30]. For the $T^{242}C$ SNP in a study of 93 patients undergoing coronary angiography, the CC homozygous were reported to have a blunted flow-dependent vasodilatation response of the epicardial coronary arteries, as compared to cumulated CT+TT genotypes[19]. This blunted response was independent of other risk factors, or

atherosclerosis itself, by multivariate analysis, suggesting that they were not secondary to coronary artery disease. However, studies in patients with hypercholesterolemia and coronary artery disease gave negative findings, thus lending no support to the contention that the CC homozygosity would imply enhanced $O_2^{\circ-}$ with ensuing diminished NO bioactivity[19].

These inconsistencies are difficult to explain; however, it should be acknowledged that all these studies comprised relatively few selected patients, thus being exposed to the chance of selection bias. Furthermore, genotyping for the *p22phox* SNP was carried out with the restriction fragment length polymorphism analysis,[19,21,23,29,30] which is affected by a considerable misgenotyping rate due to inconsistent amplicons digestion with restriction enzymes, as recently pointed out[31]. Our subjects were genotyped with a FRET technology that gives very accurate results when compared to sequencing[31]. We found a significant effect of the *p22phox* SNP *per se,* on EDV but not on EIV. Moreover, when covariates potentially affecting FBF responses were taken into consideration and examined for their potential associations with the SNP in determining EDV, significant interactions of the SNP with some conditions known to be associated with enhanced ROS generation, such as arterial hypertension, male gender and cigarette smoking were detected.

Of interest, the significant effect of the *p22phox* SNP on EDV was found, and the interactions with relevant covariates were confirmed when subjects were stratified into more homogenous subgroups according to the presence or absence of arterial hypertension not only.

Thus, these results might reconcile previous conflicting data obtained in smaller studies that might have been underpowered for detecting interactions.

Mechanisms of the blunted EDV in PH patients

A second important finding of this study, which can provide mechanistic insight into the functional role of the -930 A/G and $C^{242}T$ SNP, was provided by the L-NMMA and vitamin C experiments. Previous studies showed that inhibition of NO generation with L-NMMA blunted the FBF responses to ACH in NT, but not in PH patients, thus suggesting a negligible NO-dependent component of EDV in the latter[33,25]. As scavenging of $O_2^{\circ-}$ with vitamin C corrected the blunted EDV in PH patients, but not in NT subjects, enhanced vascular production of $O_2^{\circ-}$ causing NO destruction, was held to be the mechanism responsible for the blunted EDV in PH patients[27]. The present findings of a blunted effect of L-NMMA and enhanced FBF response to vitamin C in PH patients and of a direct strong correlation between EDV responses to inhibition of NO and scavenging of $O_2^{\circ-}$ (Fig. 12) are, therefore, fully consistent with that contention[27].
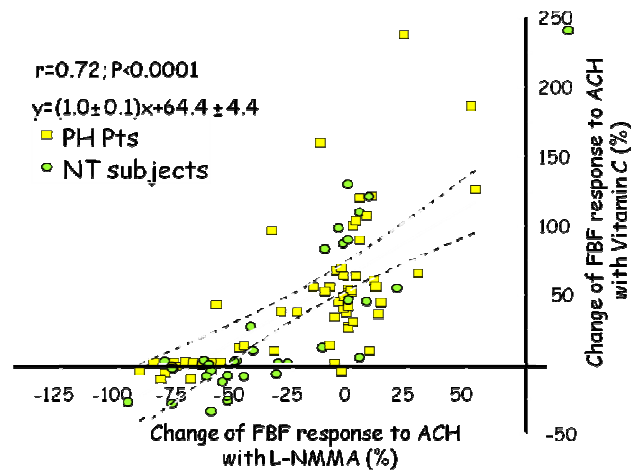

Fig. 12 The scatter plot shows a direct correlation between the changes of FBF responses to ACH during NO inhibition with L-NMMA and during scavenging of $O_2^{\circ-}$ with vitamin C. This correlation was consistently found in all p22phox C242T genotypes

Impact of the polymorphism on EDV and EIV during NO inhibition and scavenging of $O_2^{\circ-}$

Our *p22phox* SNP results add a further novel piece of information in this context. Vessel wall specimen from TT homozygous subjects were shown to have a decreased NADPH ox activity *in vitro*, as compared to the other genotypes, thus implying that this allele would carry the susceptibility to generate less $O_2^{\circ-}$ in the vascular wall. We found that NT subjects and, to a lesser extent, PH patients who were TT homozygous showed a blunted decrease of EDV after NO synthase inhibition with L-NMMA. This can suggest that a genetic predisposition to generate less $O_2^{\circ-}$ translates into an increased dependence of EDV from NO, because of a lower NO breakdown. This gene-environment interaction might have obvious important implications that should to be further explored given the involvement of $O_2^{\circ-}$ in inducing ED, CV damage and events.[34]

Our findings with vitamin C are, however, not consistent with this interpretation: the TT homozygous subjects showed a trend toward a greater, and not a lower, increase of EDV with vitamin C, particularly in the presence of PH, e.g. under conditions of enhanced $O_2^{\circ-}$ generation, but, this did not reach statistical significance.

CONCLUSIONS

Despite having a low series of patients, due to problems of recruitment of uninfected human material, the response of patients with GG/CC genotype shall not be considered accidental, since the mutation on the promoter can really change the binding capacity of the transcription factor C/EBP by modulating negatively the transcriptional activity of CYBA and mutation on the exon 4 could reduce oxidative stress[39]. These results may help identify patients at high risk of CAD, beyond the classic risk factors already known.

The present results indicate that the -930 A/G on the promoter and the $C^{242}T$ SNP of the exon 4 of the *p22phox* gene affects endothelium-dependent, but not endothelium-independent vasodilatation in humans, *per se* and via interactions with conditions that are associated with enhanced oxidative stress, as arterial hypertension, gender, and smoking. NO inhibition unveiled a significant effect of the $C^{242}T$ SNP on EDV. Thus, collectively these results indicate that the $T^{242}C$ *p22phox* SNP plays a functional role *in vivo* in humans by affecting NO bioactivity, likely by altering vascular $O_2^{\circ-}$ production.

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